App Serial # 10/054,691 Exhibit A
Yu et al. LEX-0303-USA
Novel Human Lipase and Polynucleotides Encoding the Same

1: XP_371487. phospholipase B1 ...[gi:42656279] BLink, Domains, Links

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VERSION     XP_371487.2  GI:42656279
DBSOURCE    REFSEQ: accession XM_371487.2
KEYWORDS    .
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
COMMENT     MODEL REFSEQ:  This record is predicted by automated computational
            analysis. This record is derived from an annotated genomic sequence
            (NT_022184) using gene prediction method: GNOMON, supported by mRNA
            and EST evidence.
            Also see:
                Documentation of NCBI's Annotation Process

            On Feb 19, 2004 this sequence version replaced gi:41123841.
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Jun 8 2004 17:01:12

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 FASTA searches a protein or DNA sequence data bank
 version 3.3t05 March 30, 2000
Please cite:
 W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448

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    629 residues in     1 sequences

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The best scores are:                                          opt
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Smith-Waterman score: 4011;  99.662% identity in 591 aa overlap (868-1458:39-629)

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      1080      1090      1100      1110      1120      1130
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      1440      1450
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1458 residues in 1 query   sequences
629 residues in 1 library sequences
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Function used was FASTA
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Identification of Functional Domains of Rat Intestinal Phospholipase B/Lipase

ITS cDNA CLONING, EXPRESSION, AND TISSUE DISTRIBUTION*

(Received for publication, August 8, 1997, and in revised form, October 23, 1997)

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A cDNA encoding a rat intestinal Ca^{2+}-independent phospholipase B/lipase (PLB/LIP) was cloned from an ileac mucosa cDNA library using a probe amplified by polymerase chain reaction based on the purified enzyme's sequence. PLB/LIP consists of an NH_2-terminal signal peptide, four tandem repeats of about 350 amino acids each, and a hydrophobic domain near the COOH terminus. The enzyme purified previously was found to be derived from the second repeat part. To examine the function of each domain, the full-length PLB/LIP, individual repeats, and a protein lacking the COOH-terminal hydrophobic stretch were expressed in COS-7 cells. The results showed that the second repeat, but not the other repeats, had all the activities (phospholipase A_2, lysophospholipase, and lipase) found in the purified natural and expressed full-length enzymes, suggesting repeat 2 is a catalytic domain. The full-length enzyme was mainly present in membrane fractions and efficiently solubilized by treatment with 1% Triton X-100, but not with phosphatidylinositol-specific phospholipase C. Deletion of the COOH-terminal hydrophobic stretch caused the secretion of >90% of synthesized PLB/LIP into culture media. These results suggest the hydrophobic domain is not replaced by a glycosylphosphatidylinositol anchor but serves as a membrane anchor directly. A message of the full-length PLB/LIP was abundantly expressed in the ileum and also, in a smaller, but significant amount, in the esophagus and testis. Immunohistochemistry showed that PLB/LIP is localized in brush border membranes of the absorptive cells, Paneth cells, and acrosomes of spermatid, suggesting its roles related and unrelated to intestinal digestion.

Digestion of phospholipids and triacylglycerol in gastrointestinal tract involves several hydrolysis reactions. A variety of lipases, including acid lipase in lingual gland or stomach, and pancreatic lipase (which is of primary importance in luminal digestion of fats), hydrolyze triacylglycerol to produce monoacylglycerols and free fatty acids (see Refs. 1–3 for reviews)). Pancreatic phospholipases A_2 (PLA_2)[1] hydrolyze ester bonds at the *sn*-2 position of glycerophospholipids and produce fatty acids and lysophospholipids. These steps are prerequisites for lipid absorption by intestinal epithelium cells (4). Lysophospholipid can be directly absorbed, or hydrolyzed by pancreatic lysophospholipase and converted to glycerol 3-phosphate esters and fatty acids.

Until recently, all those processes were believed to proceed in the lumen of alimentary tracts by the action of secretory enzymes mentioned above. However, recent studies suggest the presence of a lipid-hydrolyzing enzyme associated with intestinal brush border membranes (5, 6), named phospholipase B/lipase (PLB/LIP) because this enzyme displayed broad lipolytic activities (PLA_2, lysophospholipase, and lipase activities) (7). PLB/LIP might participate in terminal digestion, or membrane digestion, of dietary lipids and biliary phospholipids, like well established glycosidases and peptidases in brush border membranes (8). In the preceding paper, we detailed the purification and characterization of the catalytic domain of PLB/LIP after its solubilization from the membrane by autolysis. The purified enzyme consisted of a 14-kDa peptide and a 21-kDa glycosylated peptide and catalyzed PLA_2, lysophospholipase, and lipase reactions in a single active site (9). To understand the molecular basis of such a broad enzyme specificity, the structure of the nascent enzyme, the membrane anchoring mode, and its physiological significance, we cloned and sequenced the full-length PLB/LIP cDNA based on information of the NH_2-terminal amino acid sequences of the two peptides derived from the purified enzyme. PLB/LIP is translated as a large single peptide of 1450 amino acids containing a signal peptide, four tandem repeats, and the COOH-terminal hydrophobic stretch. The second repeat has the stretch coding the amino acid sequences found in the previously purified enzyme and has full enzymatic activities, as confirmed by the expression of individual repeats in COS-7 cells. Furthermore, to determine whether or not the hydrophobic stretch is responsible for a membrane anchor, a deleted mutant was constructed and expressed in COS-7 cells.

EXPERIMENTAL PROCEDURES

Materials—1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was obtained from Avanti Polar Lipids, Inc. Trioleoylglycerol (TOG), 1-palmitoyl-*sn*-glycero-3-phosphocholine (1-palmitoyl-GPC), and diiso-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) D63648.

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[1] The abbreviations used are: PLA_2, phospholipase A_2; PLB/LIP, phospholipase B/lipase; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; TOG, trioleoylglycerol; 1-palmitoyl-GPC, 1-palmitoyl-*sn*-glycero-3-phosphocholine; DFP, diisopropyl fluorophosphate; RT-PCR, reverse transcription-polymerase chain reaction; PCR, polymerase chain reaction; IgG, immunoglobulin G; GPI, glycosylphosphatidylinositol; DTT, dithiothreitol; PBS, phosphate-buffered saline; kb, kilobase pair(s); bp, base pair(s).

This paper is available on line at http://www.jbc.org

propyl fluorophosphate (DFP) were purchased from Sigma.

Amplification of cDNA Fragments by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Surgically removed rat intestines were washed with distilled water. The ileal mucosa was scraped off and stored at −80 °C until use. The frozen tissues (about 1.0 g) were homogenized in 10 ml of 4 M guanidine thiocyanate containing 50 mM Tris-HCl (pH 7.6), 1 mM EDTA, 2% sodium lauryl sarcosinate, and 2% 2-mercaptoethanol. The homogenate was extracted with phenol/chloroform, and RNA in the aqueous phase separated by centrifugation was precipitated with ethanol. One μg of total RNA was reverse-transcribed with 200 units of SuperScript RT (Life Technologies, Inc.) in the presence of 150 ng random hexanucleotides in a 20-μl reaction mixture according to the protocol recommended by the manufacturer. The products were heated at 70 °C for 5 min, and then a 10-μl aliquot of the reaction mixture was added to a 90-μl PCR mixture containing 100 pmol of primers. The primers used were as follows: primer KF, ccggatccAA(A/G)TT(C/T)ACITG(C/T)CCIGA(C/T)AA(A/G)GA(C/T)CC, corresponding to the previously determined peptide, KFTCPDKDP, followed by *Bam*HI adaptor; primer QT, GCIGT(C/T)TTIAT(A/G)TT(A/G)TCIGT(A/G)AANGT(C/T)TG, corresponding to the peptide QTFTDNIKTA (9), where the letter I in the oligonucleotide sequences means inosine. The PCR was conducted in 30 cycles of reactions at 94 °C for 40 s, 54 °C for 1 min, and 72 °C for 1 min. The amplified DNA fragments were visualized by ethidium bromide staining as a single band of 540 bp on a 5% polyacrylamide gel. The products were directly cloned into pCRII plasmid vector (Invitrogen) without further purification. Six clones containing the PCR products were isolated. Double-stranded DNA sequencing of each clone was performed by the dideoxy method (10) using an Applied Biosystems model 373A automatic DNA sequencer according to the manufacturer's instructions. Predicted amino acid sequences at both termini of these clones coincided with those of the primers. One clone named pCR-RIPLB was used as the probe DNA for RNA blot analysis and screening of a λ phage library.

Construction and Screening of cDNA Library—A rat ileum mucosa cDNA library was prepared based on the method of Gubler and Hoffman (11) using a λMos*Elox* cloning vector (Amersham). Six μg of poly(A)$^+$ RNA, an oligo(dT) primer, and avian myeloblastosis virus reverse transcriptase were used for the first strand synthesis. The second strand was then synthesized by treatment with RNase H and DNA polymerase I, and then the generated cDNA was blunt-ended with T4 DNA polymerase, ligated to *Eco*RI adaptor (Amersham) by T4 ligase, and further treated with polynucleotide kinase. The cDNA greater than 500 bp in length were selected on a spin column. The resultant cDNAs were ligated into an *Eco*RI site of λMos*Elox* vector, and the vectors were packaged into 3×10^6 phages. The library was screened without amplification.

For screening, 10^6 phages were plated and replicas were made from the resultant plaques (12). Positively hybridizable plaques were screened with a ^{32}P-labeled *Bam*HI cDNA fragment of pCR-RIPLB and 50 strongly hybridized clones were selected and stored at 4 °C. Randomly chosen 20 clones of the 50 were subjected to the second screening, and 19 positive clones were isolated. To recover the cloned cDNAs as plasmids, an *Escherichia coli* strain BM25.8 (Amersham) was infected with the λ phage, and plasmid was subcloned by a *cre* (site-direct endonuclease)-mediated plasmid self-subclone system (13). Plasmids prepared from ampicillin-resistant colonies were digested with *Eco*RI, and the fragments were separated on an agarose gel. Four clones revealed an identical pattern composed of three fragments of 1.4 kb, 3.1 kb, and 4.0 kb. Since the 4.0-kb fragment was derived from the vector, we concluded that each of these four clones contained a 4.5-kb insert with a single *Eco*RI site, which was the same size as the PLB/LIP transcript as judged by Northern blot analysis. One of the four clones, named pMOS-RIPLB3, was used for further analysis. To determine its complete nucleotide sequence, the *Eco*RI-digested cDNA fragments were independently subcloned into pBluescript KS(−) (Stratagene). A plasmid containing a 1.4-kb cDNA fragment (NH_2-terminal portion) and that containing a 3.1-kb fragment (COOH-terminal portion) were named pBS-3S and pBS-3L, respectively. Clones having various sizes of the insert were obtained from each plasmid by ExoIII nuclease and mung bean digestion system (Takara, Kyoto, Japan).

Preparation of Antibody and Immunoblot Analysis—Polyclonal antibodies were raised against a PLB/LIP fragment (amino acids 450–1450). The antigen was produced in *E. coli* as a fusion protein using T7 RNA polymerase expression system (14). An *Eco*RI insert of pBS-3L encoding the peptide was subcloned into *Eco*RI-cleaved pET28a(+) vector (Novagen), and it was transfected into *E. coli* strain, BL21(DE3) (Novagen). The expressed proteins were solubilized from a particulate fraction of cell homogenates with an SDS sample buffer containing 1% SDS and 10% mercaptoethanol, and subjected to SDS-PAGE (15, 16). The fusion protein (about 1.5 mg) was electrophoretically extracted from the gel slices (12). Its aliquot (about 0.4 mg) was emulsified with 2 volumes of complete Freund's adjuvant and used to immunize Japanese white rabbits (females, body weight of 2.0 kg). Immunoglobulin G (IgG) was purified from 15 ml of antiserum by an IgG purification kit (Amersham).

The freshly prepared intestinal mucosa was homogenized in 5 volumes of HBS buffer (0.25 M sucrose, 1 mM EDTA, 30 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$). Testis tissues and sperm collected from epididymis were sonicated in 10 volumes of HBS buffer, and the homogenates were centrifuged at 1,000 × *g* for 10 min at 4 °C. Supernatants were further centrifuged at 100,000 × *g* for 45 min at 4 °C, and pellets (membrane fraction) were taken and suspended in 0.1 volume of HBS buffer. All the samples were stored at −40 °C until use. The proteins (20 μg each) were subjected to SDS-PAGE (on 6 or 10% gels), and immunoblotting was performed as described (12). The antigen-antibody complexes on the membranes were further reacted with goat anti-rabbit IgG-horseradish peroxidase conjugate (Cappel). The peroxidase conjugate was developed with a Konica immunostaining kit (Konica, Japan).

Expression of PLB/LIP in COS-7 Cells—To construct the expression vector, pMOS-RIPLB3 was partially digested by *Bam*HI and the full-length 4.5-kb cDNA fragment having adaptor links on both sides was isolated by agarose gel electrophoresis and cloned into the *Bam*HI site of a pSVL vector (Pharmacia Biotech Inc.). This vector, named pSVL-RIPLB3, had an extra ATG at *Nco*I site in the adaptor sequence preceding the initiating ATG of RIPLB cDNA. This was removed by replacing an *Xho*I fragment of pSVL-RIPLB3 with a fragment from the initiator ATG to *Xho*I site (nucleotide position 333) that was amplified by PCR using the pMOS-RIPLB3 as a template and primers, Nfor (Fig. 3*b*) and L331 (CATTCTGTAGTTCGCTCTCGAGGC). The resultant vector was named pSVL-RIPLB.

Expression plasmids for repeats 1, 2, 3, or 4, named pSVL#1, pSVL#2, pSVL#3, or pSVL#4, respectively, were constructed by linking a cDNA fragment encoding the NH_2-terminal peptide of PLB/LIP including the signal peptide with a cDNA encoding each repeat to meet requirements for secretable proteins. We first constructed pSVL-Nhead that contains a cDNA encoding amino acids 1–40 of PLB/LIP and a synthetic polylinker consisting of *Nhe*I and *Dra*I sites, a stop codon, and a *Mlu*I site in this order. The insert was amplified by PCR using primers Nfor and Nrev (Fig. 3), and pSVL-RIPLB as template, and subcloned directly into a pMOS-Blue vector (Amersham). The *Apa*I-*Bam*HI fragment excised from this vector was substituted for an *Apa*I-*Bam*HI fragment of pSVL-RIPLB, generating pSVL-Nhead.

pSVL#1 containing amino acids 1–357 of PLB/LIP was generated by replacing an *Apa*I-*Dra*I fragment of pSVL-Nhead with an *Apa*I-*Pvu*II fragment of pSVL-RIPLB (Fig. 3). Expression vectors for the other repeats were constructed as follows. Fragments encoding repeats 2, 3, and 4 were first amplified by PCR using the pSVL-RIPLB template, sense primers (F2, F3, and F4, respectively) with a 5′-terminal *Nhe*I site, and antisense primers (R2, R3, and R4, respectively) with a 5′-terminal *Mlu*I and stop codon. Then they were digested by *Nhe*I and *Mlu*I, and the resultant fragments were finally cloned into pSVL-Nhead that had been previously digested by the same restriction endonucleases. An expression vector lacking the COOH-terminal hydrophobic stretch (pSVL-ΔC) was constructed by replacing the 3′-side *Bam*HI fragment of pSVL-RIPLB (nucleotides 3701–3′ end) with that of pSVL#4 (nucleotides 3701–4259).

COS-7 cells (4×10^6 cells) suspended in 0.4 ml of saline G were transfected with the plasmids (50 μg) in the presence of 250 μg of carrier DNA by electroporation using a single pulse (125 microfarads at 400 V) as reported (16). The efficiency of transfection estimated by enzyme activity recovered medium and cell fractions depended on cell density (16); the cell density of 60–70% confluence was optimal. The transfected cells were plated on two 9-cm dishes containing Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The dishes were incubated at 37 °C under an atmosphere of 5% CO_2, 95% air. The medium was changed once every 24 h. The medium was recovered for enzyme assay, and the cells were harvested for immunoblot and Northern blot analyses after 72 h.

Fractionation of COS-7 Cells—Cells were sonicated in 600 μl of HBS buffer, and the sonicate was centrifuged at 1,000 × *g* at 4 °C for 10 min. The supernatant was further centrifuged at 100,000 × *g* at 4 °C for 45 min to obtain the cytosol and membrane fractions. The pellets were resuspended in 200 μl of HBS buffer. All the fractions were stored at −40 °C until use.

Lipolytic Activity Measurements—PLA_2, lysophospholipase, and lipase activities were measured using POPC, 1-palmitoyl-GPC, and

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1 TACTCAGAGGTCCCAGACACCTGCAAGACTGAGGGATGGAGTCATGGCCAGGCGTTTCCCTGGTGGGACTGCTGCTGCTGCTACTGCTGGGACAAGGGCCCTCCCAAATCCACGGCTCT 119
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480 ATTCAGGCTAAAGAGCTGGTGAGGCATCTGAAAGACAACCCGGAACTTGATTTTGAGAAGGACTGGAAACTCATCACTGTGCTCTTCAGTAACACAAGCCAGTGCCACCTGTGTTCCTCT 599
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960 GCCCTCAGGATCTGGAATAGCATGATGGAACCAGTGGGTCGAAAGGATGGGACACTCAATGAAGCAGAGAGAAAAACAATGAAATGTCCCTCTCAGGAGAGCCCCTATCTGTTCACCTAC 1079
309 A L R I W N S M M E P V G R K D G T L N E A E R K T M K C P S Q E S P Y L F T Y 348

1080 AGAAATAGCAACTACCAGGCCAGACAGCTGAAACCCATAGGAAAGTTTCAGATGAAAGAAGGAACAAAATTCACCTGTCCTGACAAAGACCCCTCGGACTCCATCCCCACAACAGTTCAC 1199
349 R N S N] Y Q A R Q L K P I G K F Q M [K E G T K F T C P D K D P S D S I P T T V H 388
repeat 2
1200 AGGCTGAGGCCGGCTGACATCAAGGTCATCGGAGCCATGGGTGACTCGCTCACGGCAGGCAACGGGGCAGGGTCCAGCCCTGGGAATGTCTTGGATGTCTTAACTCAATACCGAGGCCTG 1319
389 R L R P A D I K V I G A M G D S L T A G N G A G S S P G N V L D V L T Q Y R G L 428

1320 TCGTGGAGTGTGGGCGGAGATGAGACCATCGAGACCGTGACCACCCTAGCCAACATCCTCCGGGAATTCAACCCCTCCTTGAAGGGCTTCTCTGTTGGCACTGGGAAAGAAAACACTCCC 1439
429 S W S V G G D E T I E T V T T L A N I L R E F N P S L K G F S V G T G K E N T P 468

1440 CGAGCATCCTTCAACCAGGCCGTAGCAGGAGCCAAATCTGATGGCTTAGCTGCCCAGGCCAAAAAGCTGGTGAGCCTGATGAAGGATGACAAGACAATAAACTTTCAGGAAGACTGGAAG 1559
469 R A S F N Q A V A G A K S D G L A A Q A K K L V S L M K D D K T I N F Q E D W K 508

1560 ATAATCACTGTGTTTATAGGAGGCAACGACCTCTGTGGCTCCTGCAATAACCTGGCTCGCTTTTCTCCCCAAACCTTCACAGACAACATCAAGACGGCCCTGGACATCCTCCATGCAGAG 1679
509 I I T V F I G G N D L C G S C N N L A R F S P Q T F T D N I K T A L D I L H A E 548

1680 GTTCCCCGGGCCTTTGTGAACATGGTCTCGGTGATTGAGATCACCCCCTTGAGAGAACTGTTCAATGAACCTAAAGTCAGCTGCCCACGGATGATCCTCAGGAGCCTGTGTCCTTGTGTC 1799
549 V P R A F V N M V S V I E I T P L R E L F N E P K V S C P R M I L R S L C P C V 588

1800 TTGAACCTTGGTGAGAACTCAGCAGAACTTGCCCAACTTGTGGAAAGGAACAGGCAGTATCAGGAAGAAACTGGAAAACTGATTGAGAGTGGCCGATACGACACCAGGGATGACTTCACC 1919
589 L N L G E N S A E L A Q L V E R N R Q Y Q E E T G K L I E S G R Y D T R D D F T 628

1920 GTGGTCCTCCAGCCCATGTTTGAAAATGTCGTCATGCCACGGACCCTGGAGGGCTTGCCCGACAGCTCTTTCTTTGCCCCTGACTGTTTCCACTTCAATGTCAAGACTCACGCTCGCTCA 2039
629 V V L Q P M F E N V V M P R T L E G L P D S S F F A P D C F H F N V K T H A R S 668

2040 GCCATCGCCCTCTGGAAGAACATGCTAGAACCTGTGGGCCGCAAGACAAGACATCAGAATTTTGAAATCAAGGTCCCTATCATGTGTCCAAACCAGACCTCACCGTTTCTGAGCACCACC 2159
669 A I A L W K N M L E P V G R K T R H Q N F E I K V P I M C P N Q T S P F L S T T 708

2160 AAGAACAGCAACCTGGGACATGGAACTTCGATGTCTTGTGAGGAGAAAGCCCCCTCTGCCTCACCACCAACCTCAGTGCATACCCTGAGACCTGCAGACATCCAAGTTGTGGCAGCTCTG 2279
709 K N S N] L [G H G T S M S C E E K A P S A S P P T S V H T L R P A D I Q V V A A L 748
repeat 3
2280 GGAGACTCTGTGACTGCTGGCAATGGAATCAGCTCCCAAGAAGGTGATCTCGCTGATGTTACCACACAGTATCGAGGACTGTCCTACAGTGCTGGTGGGGACAAGTTCCTGGAGAATGTG 2399
749 G D S V T A G N G I S S Q E G D L A D V T T Q Y R G L S Y S A G G D K F L E N V 788

2400 ACCACCTTGCCCAACATCCTCCGGGAATTTAATGGAAATCTCACAGGCTACTCAGTCGGAACCGGTGACGTCAACTCTGCAAGCGCGTTCCTTAACCAGGCTGTCCCTGGGGCAAAGGCT 2519
789 T T L P N I L R E F N G N L T G Y S V G T G D V N S A S A F L N Q A V P G A K A 828

2520 GAGAACCTTGCAAGTCAAGTCCAGACTCTGATTCAGAAGATGAAGAATGACACCAGAGTGAACTTTCACCAAGACTGGAAGGTCATCACTGTGATGATTGGGGCCAGCGACTTGTGTGAC 2639
829 E N L A S Q V Q T L I Q K M K N D T R V N F H Q D W K V I T V M I G A S D L C D 868

2640 TTCTGCAAGGATTCGAATCGTTACTCTGCAGCCAATTTTTCTGACCATCTCCGCAATGCCTTGGACATCCTGCATAAGGAGGTACCCAGAGCCCTGGTCAACCTTGTGGACTTCATGAAC 2759
869 F C K D S N R Y S A A N F S D H L R N A L D I L H K E V P R A L V N L V D F M N 908

2760 CCCAGTATCATTCGGCAAGTGTTCCTGAAGAACCCAGACAAGTGCCCTGTGAATCAGACCAGTGTCCTGTGCAACTGTGTTCTGACCCCAGGGGAGGATTCCCATGAGCTGGCAAGGTTG 2879
909 P S I I R Q V F L K N P D K C P V N Q T S V L C N C V L T P G E D S H E L A R L 948

2880 GAGGCCTTCACCAAATCCTACCAGAGTAGCATGCTTCAACTGGTTGAGTCAGGCCGCTACGACACCCGGGAGGATTTCTCTGTGGTACTGCAGCCCTTTCTCTTCAACATCAGGCTCCCC 2999
949 E A F T K S Y Q S S M L Q L V E S G R Y D T R E D F S V V L Q P F L F N I R L P 988

3000 ATCCTAGAGAATGGGAATCCAGATACATCCTTCTTTGCCCCAGACTGCATCCTCCTAAGCCAGAAGTTCCACACTCAGCTCGCGAGAGCCCTTTGGGCCAATATGCTTGAACCCCTGGGA 3119
989 I L E N G N P D T S F F A P D C I L L S Q K F H T Q L A R A L W A N M L E P L G 1028

3120 AAGAAAATGGATACTTTGGACCCGAAAGAACTCATAGCTTTGGCCTGCCCCACCAAGGACAAGCCCTTCCTGAGAACCTTCCGGAACAGTAACTACACGTACCCTATCAAGCCAGCCATT 3239
1029 K K M D T L D P K E L I A L A C P T K D K P F L R T F R N S N] Y T Y P I K P A I 1068

3240 GAGAATTGGGGCAGTGACTTCCTGTGCACAGAGCAGAGTCCTTCCAGCAAGGTACCCACCTCAGTTCATGAGCTCCGACCATCAGACATCAAGGTGGTGGCAGCAATGGGTGACTTTCTG 3359
1069 E [N W G S D F L C T E Q S P S S K V P T S V H E L R P S D I K V V A A M G D F L 1108
repeat 4
3360 ACGACAGCCACCGGAGCTCGACCAAGTGAGTCCAGCAGTCTAGACACCCCCTGGAGGGGGCTGTCTTGGAGCATTGGAGGAGATGGAACGTTGGAGACCCATACCACACTGCCCAACATC 3479
1109 T T A T G A R P S E S S S L D T P W R G L S W S I G G D G T L E T H T T L P N I 1148

3480 CTGAAGAAGTTCAACCCTTCCATCCTTGGATTCTCCACCGGTACCCTGGAGAACACGGCAGGATTAAATGTGGCAGAAGAAGGCGCCAGAGCTCAGGACATGCCGGCCCAGGCTCAGGCC 3599
1149 L K K F N P S I L G F S T G T L E N T A G L N V A E E G A R A Q D M P A Q A Q A 1188

3600 CTGGTGAAGAAGATGAAAAGCACCCCTACAATCAACATACAGGAAGACTGGAAGCTGATTACACTCCTCATCGGGAACAACGACCTGTGTCTTTACTGTGAGGATCCGGAGAACTACTCA 3719
1189 L V K K M K S T P T I N I Q E D W K L I T L L I G N N D L C L Y C E D P E N Y S 1228

3720 ACCAGGGAGTATGTCAAGTACATCCAGCATGCCTTGGACATCTTCTATGAGGAGCTTCCCAGGGTTTTCATCAACGTGGTGGAAGTCATGGAGCTGTCCGGTCTGCTCCACGACCAGGGC 3839
1229 T R E Y V K Y I Q H A L D I F Y E E L P R V F I N V V E V M E L S G L L H D Q G 1268

3840 GGGAAATGTGCCATGCCGTTGGCTGTCCAGAAAAACTGCAGTTGCCTTAAACGCTCTCAAAACCTCATGGCAATGCAGGAGCTGAAGAAAGTCAACGGGAACCTCCAGAGTGCCCTCTCG 3959
1269 G K C A M P L A V Q K N C S C L K R S Q N L M A M Q E L K K V N G N L Q S A L S 1308

3960 GAGCTCTCCTACTGGCATCGGTACATGCAGCGTGAGGACTTTGCAGTCACTGTCCAGCCTTTCTTCCGGAATACCTTTGTCCCACTGGATGAGCGTGGGGGCCTCGACCTCACTTTCTTC 4079
1309 E L S Y W H R Y M Q R E D F A V T V Q P F F R N T F V P L D E R G G L D L T F F 1348

4080 TCTGAAGACTGTTTCCACTTCTCAGTCCGTGGGCATGCTGAGATGGCCATTGCCCTCTGGAACAACATGCTGGAACCAGTGGGCAAGAAGACAACCTCCAATAACTTCACATACAACCGG 4199
1349 S E D C F H F S V R G H A E M A I A L W N N M L E P V G K K T T S N N F T Y N R 1388

4200 ACCAAACTCAAGTGTCCCTCGCCTGAAAACCCTTTCCTCTACACTGTCCGGAACAGTCAGATTCTTCTAGACAAGGCTAAAGAAAACTCCAATACACTCTACTGGGCAGTGCCAGTGGCT 4319
1389 T K L K C P S P E N P F L Y T V R N S Q] I L L D K A K E N S N T [L Y W A V P V A 1428

4320 GCAGTAGGTGGCCTGGTAGTTGGCATCCTTGGAATGATGTTGTGGAGAACTGTGAGACTCGTCCAATAGAAGGAGGAGGAGACTTTTCCAAATACAAGTGTGGACCTTGAGTTGACCCAG 4439
1429 A V G G L V V G I L G M M L W] R T V R L V Q 1450

4440 GGATGCTGTTTCAGAGGAGAAGCTCAGAGATTGACCCTTGGTACCAGACAGCAATTACGTAATCTCAGGGATGGGGACTGCGTGCCTTTCTCTCTTGGGAGTTCACCATCTTCTTTTGGA 4559
4560 AAGATAAATAAGAAAGAGTGGCTACATCAGT(A)n
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FIG. 1

triolein as substrates, respectively, as described in the preceding paper (9). The lipolytic activities of PLB/LIP toward exogenous substrates were expressed as the difference between the activities in the presence and absence of the substrates and as nanomoles of free fatty acid released/1 min. Protein concentration was measured by a Bio-Rad protein assay system.

RT-PCR Analysis—Total RNA (1 μg) prepared from the ileum and poly(A)-rich RNA (1 μg) prepared from esophagus or testis were reverse-transcribed as described above. Transcripts (4 μl) were mixed with a pair of primers (50 pmol each), Nfor and R2 or F3 and R4, in 5 μl of 10 × *Taq* polymerase buffer supplied by company (Takara), 4 μl of 10 mM dNTP solution, 5 μl of dimethyl sulfoxide, and H_2O to give the final volume of reaction mixture, 50 μl. The mixture was overlaid with 30 μl of mineral oil to prevent evaporation. *Taq* polymerase (2.5 units) was added to the reaction mixture, and the chain reaction was conducted as follows: denaturation at 94 °C for 40 s, annealing at 60 °C for 1 min, and polymerization at 72 °C for 4 min with 30 cycles.

Immunohistochemical Analysis—Rat ileum and testis were fixed in 4% paraformaldehyde/PBS (pH 7.4) for 2 h. Frozen sections (6 μm) prepared on glass slides were stained immunohistochemically (17). Prior to incubation with the primary antiserum, the sections were treated with Gomori's oxidation mixture (0.5% potassium permanganate, 0.25% sulfuric acid) (18). The primary antiserum was diluted 1:300, and the second antibody (fluorescein isothiocyanate-labeled goat anti-rabbit IgG) was diluted 1:300. For the controls, the preimmune rabbit serum and the primary antiserum absorbed by the *E. coli* extract containing overexpressed antigen (PLB/LIP) were used. The *E. coli* containing PLB/LIP was solubilized in 1% SDS-PBS, diluted 20 times with PBS, and incubated with the antiserum for 12 h at 4 °C. The specimens were observed under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). For testes, photographs were taken in combination with phase-contrast transmission light.

RESULTS

Isolation of cDNA Encoding PLB/LIP—A set of oligonucleotide primers designed on the basis of previously determined partial amino acid sequences of rat intestinal PLB/LIP (9) was used for amplification of cDNA fragments from total RNA of intestinal mucosa by RT-PCR. The PCR products were visualized as a clear 540-bp single band on agarose or polyacrylamide gel. The cDNA was sequenced, and parts of the predicted amino acid sequence coincided with those of peptides derived from the purified enzyme. The cDNA was used as a probe for screening the cDNA library of ileal mucosa. Four independent clones were isolated, all having the identical fragment pattern when digested by restriction enzymes. A cDNA insert of one of the clones was cleaved in two fragments by *Eco*RI digestion, each of which was subcloned into plasmid and partially sequenced. A longer fragment had a poly(A) tail and the sequence corresponding to the large peptide of purified PLB/LIP, and the other had a TGA-opal codon just six bases upstream of the first ATG starting codon and the sequence corresponding to its small peptide. From these results, we concluded that this clone contained a full open reading frame of PLB/LIP.

Nucleotide Sequence and Predicted Amino Acid Sequence—As shown in Fig. 1, the full-length 4590-bp cDNA of PLB/LIP contained an open reading frame encoding a 1450-amino acid protein with a calculated molecular mass of 161 kDa and a potential polyadenylation sequence (AATAAn) (19) just upstream of its poly(A) tail. Two hydrophobic regions were identified in the protein: the first was a putative 30-amino acid signal peptide at the NH_2 terminus (20), and the second a stretch from residue 1421 to 1443, functioning as a transmembrane anchor as described below. Notably, the PLB/LIP contains four tandem repeats in a putative extracellular domain of the protein, each repeat being composed of about 350 amino acid residues (Fig. 2). These repeats began shortly after the signal sequence and occupied 93% of the entire protein. Amino acid sequences corresponding to the PCR-amplified cDNA probe were found in the second repeat.

A Swissprot data base search revealed that the amino acid sequence of PLB/LIP was 67.9% identical to that of AdRab-B, the cDNA of which had been isolated as an adult-specific clone of rabbit intestine (21). Both proteins shared functionally important domains, *i.e.* a putative NH_2-terminal signal sequence, four tandem repeats each of which was composed of about 350 amino acids and occupied more than 90% of the entire protein, and a short hydrophobic stretch followed by a short hydrophilic one at the COOH-terminal end. There are 14 potential *N*-glycosylation sites, of which one is in the NH_2-terminal sequence preceding repeat 1, two in repeat 1, one in repeat 2, six in repeat 3, and four in repeat 4, when weak sites in an Asn-Pro-Ser(Thr) sequence were not counted.

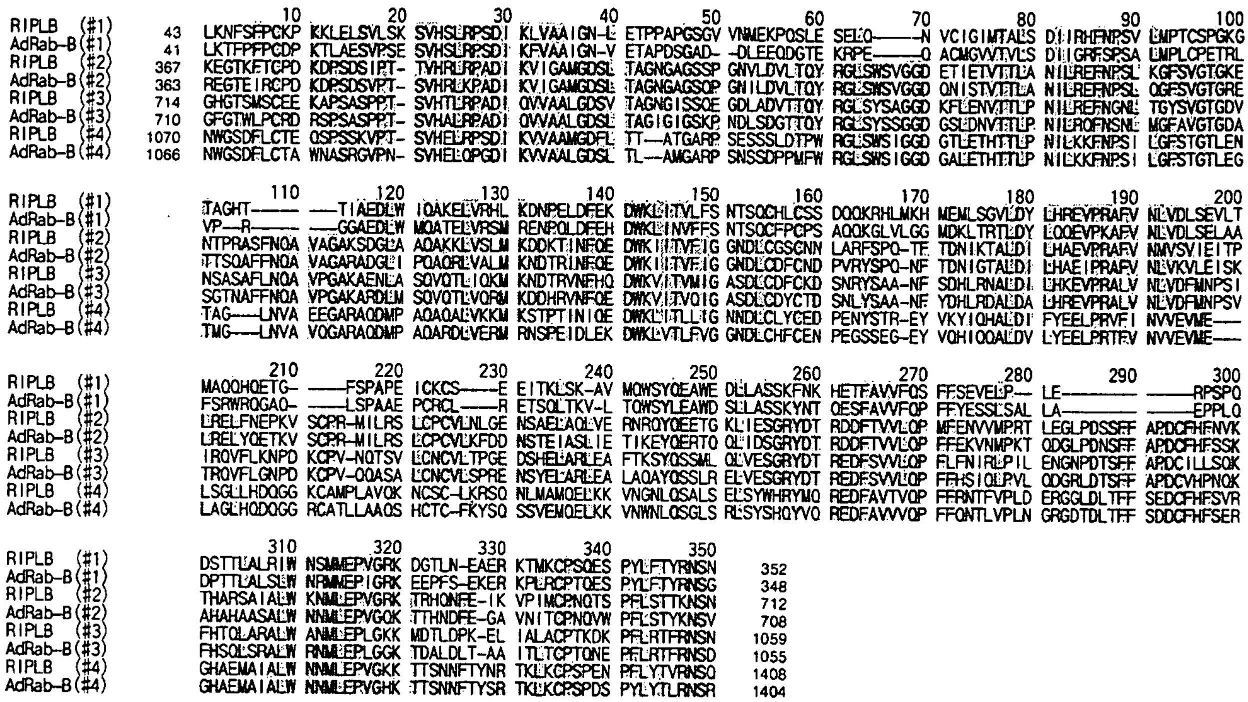
Expression of PLB/LIP in COS-7 Cells—To confirm that the rat intestinal PLB/LIP cDNA determined here encodes an active enzyme, we expressed the full-length cDNA in COS-7 cells. The cells and culture media were separately recovered and the former was further fractionated into cytosol and membrane fractions as described under "Experimental Procedures." Expression was assessed in these fractions by enzyme assay and immunoblot analyses. The cells transfected with the recombinant plasmid revealed greatly enhanced PLA_2, lysophospholipase and lipase activities, compared with cells transfected with the pSVL vector alone. This confirmed that the full-length PLB/LIP exhibits the same substrate specificity as the enzyme purified from rat intestine (9) as a single polypeptide protein. These activities were mainly localized in membrane fractions as verified by immunoblot analysis (Fig. 4, *a* and *b*), confirming PLB/LIP is a membrane-bound protein (9). The enzyme produced by COS-7 cells apparently migrated as two bands on a 6% gel, and their estimated molecular masses (~200 and 220 kDa) were larger than that calculated from the predicted amino acid sequence data (161 kDa). This may be due in part to the difference in the degree of glycosylation.

Membrane Anchoring Mode of PLB/LIP—The COOH-terminal segment, residues 1421–1443, is the only stretch that is hydrophobic and long enough to meet requirement for membrane anchoring. To determine whether the stretch plays a role in binding of the enzyme to membrane, an expression vector (pSVL-ΔC) that lacked the COOH-terminal 42 amino acid residues (Fig. 3*c*) was constructed and introduced into COS-7 cells. Immunoblot analysis indicated that about 90% of the expressed truncated enzyme was recovered in culture media (Fig. 4*a*), in contrast with membrane localization of the full-length PLB/LIP. In that culture medium, PLA_2 and lysophospholipase activities were unexpectedly not observed, but a substantial activity of lipase was detectable. The truncated protein, however, restored phospholipid hydrolyzing activities to the level as comparable as lipase activity, when dithiothreitol (DTT) was added to the reaction mixtures to the final concentration of 4–20 mM. This apparent activation of PLA_2 and lysophospholipase activities occurred only in the truncated enzyme released in culture media. DTT (4 mM) treatment had little effect on the activities of the membrane-bound PLB/LIP.

There are two possibilities that the COOH-terminal hydro-

FIG. 1. **Nucleotide sequence of PLB/LIP cDNA and its predicted amino acid sequence.** Predicted amino acid sequence of PLB/LIP is shown just below each line of the nucleotide sequence. A putative signal peptide is indicated by a *dashed underline*, and a membrane anchoring region is *boxed*. The four tandem repeats are *bracketed* and labeled. The NH_2-terminal amino acid sequences of the large and small fragments of the purified enzyme are *underlined*. The *asterisks* indicate the potential *N*-glycosylation sites, in which a weak site, an Asn in the NPS(T) sequence, at positions 123, 452, 908, or 1153 is excluded.

a



b

	Identity (%)			
	#1	#2	#3	#4
RIPLB vs AdRab-B	41.5	68.7	66.1	67.8

c

RIPLB	Identity (%) #1	#2	#3	#4
#1		28.8	27.1	27.1
#2	28.8		50.4	43.6
#3	27.1	50.4		38.7
#4	27.1	43.6	38.7	

FIG. 2. **Alignment of four tandem repeats of PLB/LIP and AdRab-B.** *a*, when more than four amino acids are identical at each position, they are *shaded*. The numbers from the initiator Met are indicated in the NH_2 and COOH termini of each repeat. *b*, sequence identity between corresponding repeats of PLB/LIP and AdRab-B. *c*, a pairwise comparison of all repeats of PLB/LIP as regards sequence identity.

phobic stretch can serve as a direct membrane anchor or as a signal for attaching a glycosylphosphatidylinositol (GPI) moiety to the COOH-terminal end after the cleavage of that stretch (22). As in the case of PLB/LIP in brush border membranes (9), phospholipase C treatment (25 and 100 munits/ml) did not solubilized PLA_2 activity in membrane fractions of full-length PLB/LIP-expressing COS-7 cells, but treatment with 1% Triton X-100, a poor solubilizer for GPI-anchored proteins (23), did solubilize 72% of the activity. This suggests that the hydrophobic stretch functions as a direct membrane anchor.

Location of the Catalytic Domain—The enzyme purified from rat intestine had an apparent molecular weight of 35,000 with PLA_2, lysophospholipase and lipase activities. It consisted of the 14-kDa and 21-kDa peptides, and their amino acid sequences were derived from the second repeat of PLB/LIP, suggesting the second repeat is a catalytic domain. To determine which domains are responsible for the activities observed in the expressed full-length and purified natural PLB/LIP, we constructed four expression vectors, each of which contained a nucleotide sequence encoding a NH_2-terminal signal peptide and the respective repeats (Fig. 3c), and transfected them into COS-7 cells. The antibody used in this study (raised against the PLB/LIP peptide, which lacked the first repeat) immunoreacted with the second and third repeats well, but did not with the first or the fourth repeat. We estimated the level of expression of each repeat in the cells by Northern blot analysis using the full-length PLB/LIP cDNA as a probe. As shown in Fig. 5*a*, transcripts of 2.0 kb were found at comparable levels in the cells transfected with each vector. The culture media and harvested cells were examined for the enzyme activities (Fig. 5*b*). All the activities found in the purified enzyme were present in the culture media of repeat 2-expressing cells, but not in the media of cells transfected with the other constructs. No activity was detected in the cell homogenates except for a trace amount of enzyme activities in repeat 2-expressing cell fractions (data not shown). This may preclude impaired secretion of expressed proteins. Furthermore, dithiothreitol had no effect on the enzyme activities of all culture media (data not shown). DFP is a potent inhibitor that inhibited PLA_2, lysophospholipase, and lipase activities of the purified PLB/LIP to a comparable extent (9). It similarly inhibited these three activities of the expressed full-length PLB/LIP by 99, 88, and 94%, respectively, and of the repeat 2 enzyme by 99, 97, and 91%, respectively, compared with those in the absence of inhibitors. These results suggested

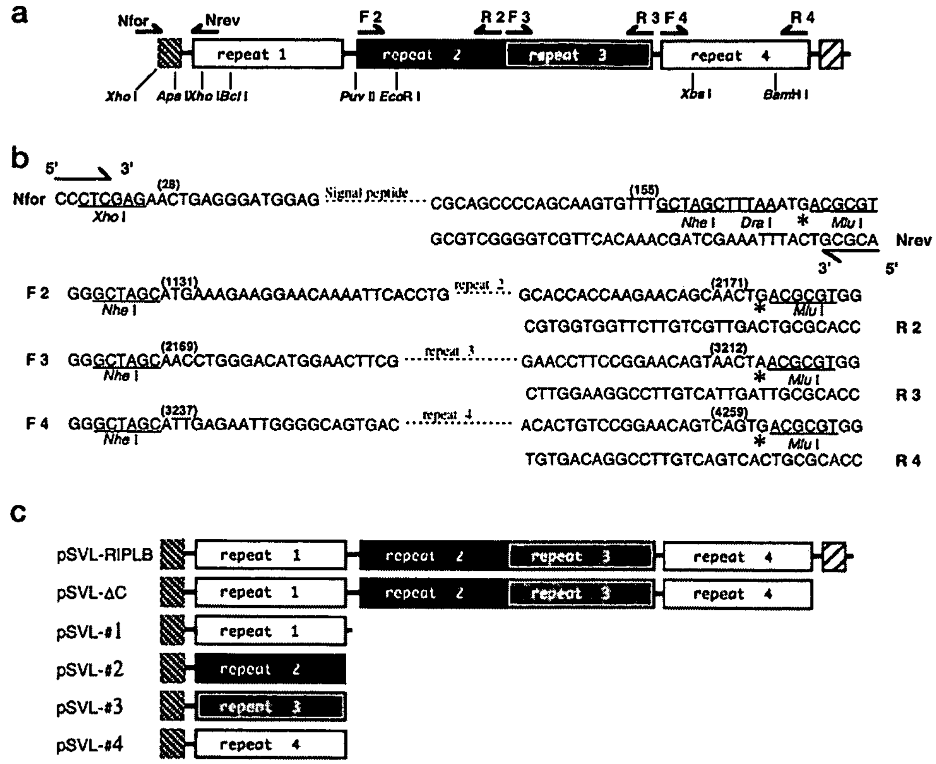


FIG. 3. **Constructions used in expression of rat intestinal PLB/LIP.** *a*, oligonucleotide primers used for PCR (*hemi-arrows*), and restriction enzyme sites are indicated along an outlined structure of pSVL-RIPLB, and procedures of preparing pSVL-Nhead are summarized: box with *thick diagonal stripes*, the signal peptide; box with *thin diagonal stripes*, the hydrophobic stretch. *F* and *R* indicate sense and antisense primers, respectively. *b*, sequences of the primers. The restriction enzyme sites connected with the 5′-end of each primer are *underlined*. *c*, expression vectors were prepared using pSVL-Nhead and pSVL-RIPLB as described under "Experimental Procedures."

that repeat 2 is the catalytic domain with a single active site responsible for all the enzymatic activities tested.

Distribution of PLB/LIP—The distribution of PLB/LIP among various rat tissues was determined by Northern analysis using rat PLB/LIP cDNA as a probe. Fig. 6*a* showed that two kinds of transcripts of about 4.6 kb and larger than 6.0 kb in length were readily detected in the ileum. The shorter transcript was also present in esophagus and testis in a small amount. Further examination of intestinal segments showed that a large amount of message existed in the ileum and a trace amount in jejunum, but not in duodenum (Fig. 6*b*). No difference was found in the levels of the message between upper and lower portions of esophagus. The message was exclusively expressed in the esophageal mucosa (Fig. 6*c*).

To ascertain that the 4.6-kb transcripts present in esophagus and testis were identical to that in intestine, the size of transcripts in these tissues was analyzed by RT-PCR. Because the full-length mRNA was too large to be amplified by PCR, we amplified two fragments from the message separately; one corresponded to repeats 1 and 2, and the other to repeats 3 and 4 (Fig. 7) as described under "Experimental Procedures." Since the amount of messages in esophagus and testis seemed to be less than 2% of that in the ileum, poly(A)-rich fractions prepared from the former two tissues were used for reverse transcription. As shown in Fig. 7 (*a* and *b*), single bands of the expected sizes (2164 or 2109 bp, respectively) in all three tissues were detected at the identical position. To eliminate a possibility that these PCR bands were amplified from genomic DNA contaminated into the RNA fractions, a negative control reaction was performed with 1 μg of rat genomic DNA. No specific DNA fragment was amplified from genomic DNA under these conditions (data not shown). The PCR products were cloned into plasmid vectors, and their nucleotide sequences were determined up to about 300 bp from both the termini. The cDNAs of esophagus and testis were found to have sequences identical to that of the intestine cDNA. Furthermore, digestion of the PCR products of all tissues by restriction enzymes gave the identical fragment patterns on agarose gel electrophoresis (Fig. 7). These results strongly suggest that the 4.6-kb transcripts expressed in esophagus and testis are identical to the intestinal PLB/LIP mRNA.

Next, we examined levels of protein expression in the mRNA-expressing tissues by immunoblot analysis using SDS-PAGE under the reducing conditions. As shown in Fig. 8, the immunoblot of freshly prepared ileac mucosa preparations revealed at least three strongly stained bands corresponding to the molecular masses of ~200, 130, and 90 kDa. PLB/LIP was not detectable in testis homogenate, but a faint band of ~200 kDa, which is the same as the largest one of the three bands found in ileum homogenate, was detectable in membrane fractions of testis (Fig. 8). When membrane fractions of sperm collected from epididymis were used for the analysis, the single band appeared more clearly. This band was not detectable using preimmune serum (not shown), suggesting that the PLB/LIP protein is expressed in the membrane fractions of sperm (and its precursor). Comparison of the sizes of PLB/LIP in freshly prepared extracts of the ileum, sperm membrane, and COS-7 cells suggested that proteolytic cleavage was considerably limited in the latter two extracts.

We investigated the localization of PLB/LIP in the ileum and testis by light microscopy using anti-PLB/LIP IgG. In the ileum, absorptive cells located in the apical region of villi were diffusely but strongly stained by anti-PLB/LIP IgG (Fig. 9*a*). Examination on higher magnification showed that the brush border membranes were more intensely stained than the cytoplasm (Fig. 9*c*). No immunopositive reactions were observed in

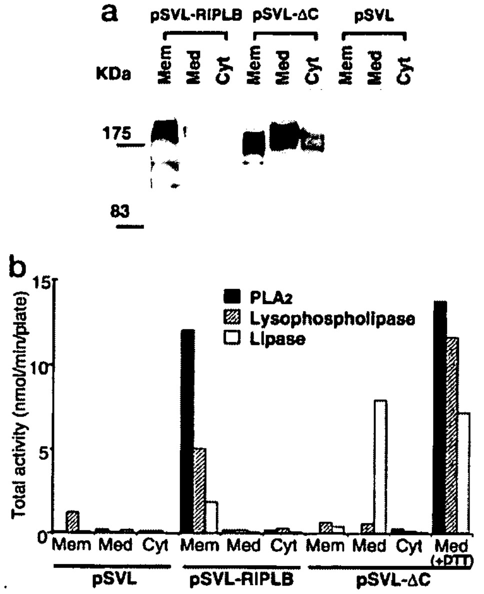


FIG. 4. **Effects of deletion of a COOH-terminal hydrophobic stretch on subcellular localization of PLB/LIP expressed in COS-7 cells.** *a*, Immunoblotting of PLB/LIP in membrane (*Mem*), culture medium (*Med*), and cytosol (*Cyt*) fractions of COS-7 cells transfected with the wild-type pSVL-RIPLB, deleted pSVL-ΔC (refer to Fig. 3c), or vector alone using the polyclonal anti-PLB/LIP IgG after SDS-PAGE on a 6% gel. The protein amounts applied to *Mem* and *Cyt lanes* were 10 times as much as to *Med lanes*. *b*, lipolytic activities were determined for each fraction as described under "Experimental Procedures." The substrate used were POPC, 1-palmitoyl-GPC, and TOG (1 mM each). The activities in culture media of deleted mutant expressing cells were assayed in the presence and absence of 4 mM DTT.

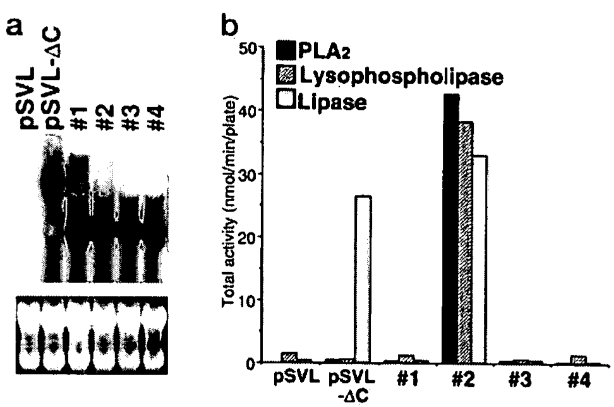


FIG. 5. **The second repeat is a catalytic domain.** *a*, Northern blot analysis of PLB/LIP mRNA extracted from cells transfected with the same constructs as in Fig. 3c. *Upper panel*, total RNA (20 μg) was electrophoresed, blotted onto a nylon membrane, and hybridized with the [^{32}P]dCTP-labeled PLB/LIP cDNA fragment that had been prepared by PCR amplification from the pMOS-RIPLB3 plasmid with Nfor and R4 primers (corresponding to almost full-length RIPLB, see Fig. 3*a*). *Lower panel*, ribosomal RNAs were stained by ethidium bromide. *b*, lipolytic activities in each culture medium were tested as in Fig. 4*b*. As a positive control, we used the culture medium of COS-7 cells transfected with pSVL-ΔC, because the expressed full-length enzyme was not secreted into the medium. Only the lipase activity was detected in this medium in the absence of DTT as in Fig. 4*b*.

control sections stained with the antibody preabsorbed with *E. coli* extracts containing the PLB/LIP fragment as antigen (Fig. 9*b*). Secretory granules of Paneth cells were also stained (Fig. 9*d*). In testis, spermatocytes and spermatids were stained with the antibody, being visualized as round and crescent bodies, respectively (Fig. 9*e*), but not with preimmune serum (Fig. 9*f*). The combination of the immunofluorescent and phase-contrast

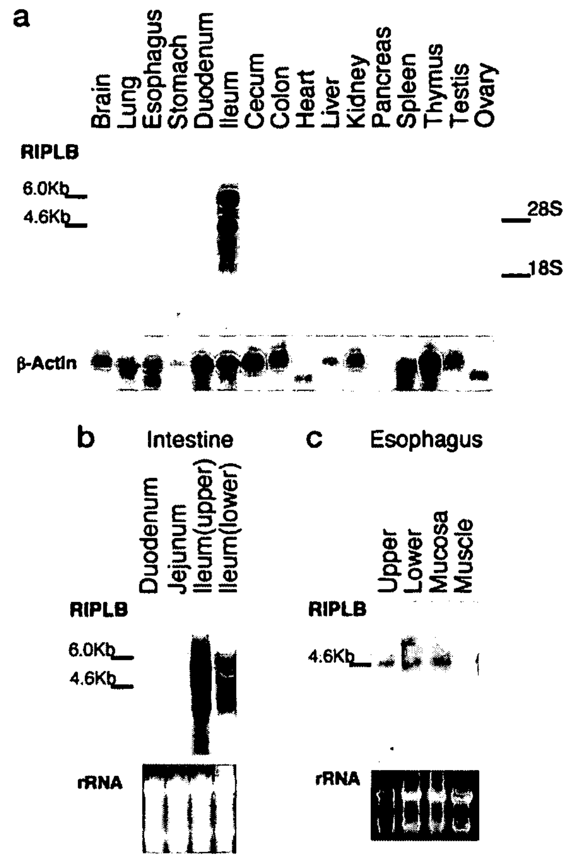


FIG. 6. **Distribution of PLB/LIP mRNA in various rat tissues.** Northern blot analysis was performed using a *Bam*HI fragment of pCR-RIPLB (*upper panels*) or an actin cDNA fragment (*a, lower panel*) as a probe. In *a* and *b*, each lane contained 30 μg of total RNA, and in *c* 20 μg of total RNA. *b*, the small intestine was divided into four parts: duodenum, jejunum, and upper and lower ileum. *c*, the esophagus was cut into the upper and lower halves, and total RNA was extracted from the whole tissue. In some cases, the mucosal layer was scraped off with forceps after the tissue was inverted, and total RNA was extracted from the scrapings and the remaining part containing muscle and adventitia.

figures showed that PLB/LIP proteins may be localized in the acrosome.

DISCUSSION

Using a probe amplified by RT-PCR based on the knowledge of partial amino acid sequences of the previously purified PLB/LIP (9), we isolated and sequenced its full-length cDNA. The NH_2-terminal amino acid sequences of the small and large fragments of purified PLB/LIP were found in complete accordance with the second repeat part of the deduced amino acid sequence. COS-7 cells transfected with the full-length cDNA produced the active enzyme that exhibited the same substrate specificity, *i.e.* PLA_2, lysophospholipase, and lipase activities, as the purified enzyme (9). These results demonstrated that the isolated cDNA indeed encodes the functional rat intestinal PLB/LIP.

The amino acid sequence of PLB/LIP showed 67.9% similarity to that of rabbit AdRab-B protein that had been isolated from an adult intestine specific cDNA library (21). Both proteins had the primary structural arrangement similar to lactose-phlorizin hydrolase, a well characterized hydrolase associated with brush border membranes: a signal peptide, four internal repeats, and a COOH-terminal transmembrane anchor. This suggests AdRab-B is a rabbit counterpart of rat PLB/LIP, although their substrate specificities were different from each other (9). Pind and Kuksis (24) reported a purifica-

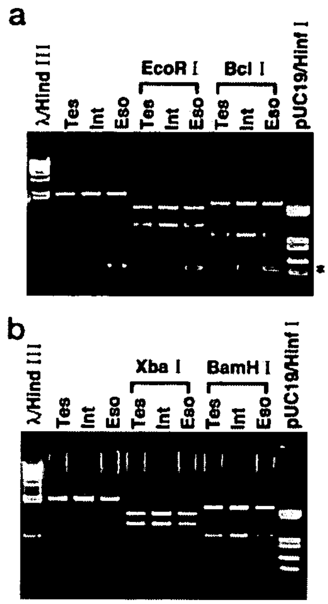


FIG. 7. **RT-PCR analysis of PLB/LIP transcript.** Poly(A)-rich RNA (1.0 μg) from the testis (*Tes*) and esophagus (*Eso*) or 0.1 μg of total RNA from the intestine (*Int*) was reverse-transcribed with random hexanucleotides. The resultant single strand cDNAs were used for PCR amplification of cDNA encoding repeats 1 and 2 with primers Nfor and R2 (*a*) and of that encoding repeats 3 and 4 with primers F3 and R4 (*b*). After purification by phenol extraction, aliquots of the former products were digested with *Eco*RI or *Bcl*I, while those of the latter were digested with *Xba*I or *Bam*HI (see Fig. 3). The digests were separated on a 1.2% agarose gel. All digestions gave two fragments of the following lengths: 796 and 1369 bp for *Eco*RI digestion, 582 and 1582 bp for *Bcl*I digestion, 870 and 1239 bp for *Xba*I digestion, 570 and 1541 bp for *Bam*HI digestion. The sizes of markers are: 23.13, 9.24, 6.56, 4.36, 2.32, 2.02, and 0.56 kb for λ/*Hin*dIII; 1419, 517, 396, 214, and 75 bp for pUC19/*Hin*fI. *Asterisk* denotes a nonspecific product, which was also amplified from rat genomic DNA.

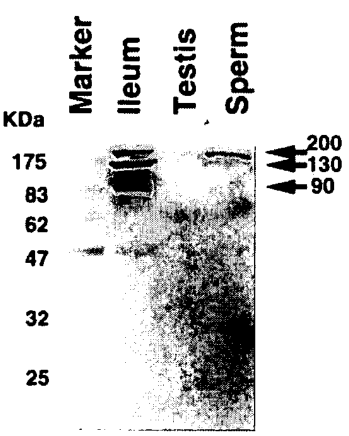


FIG. 8. **Immunoblot analysis of PLB/LIP in the ileum, testis, and sperm.** Ileum (3 μg) and testis (20 μg) homogenates and sperm membrane fractions (20 μg) were subjected to SDS-PAGE on a 12% gel, blotted to a PVDF membrane, and detected with 500-fold diluted anti-PLB/LIP serum. Prestained proteins (New England Biolabs) were used as size markers.

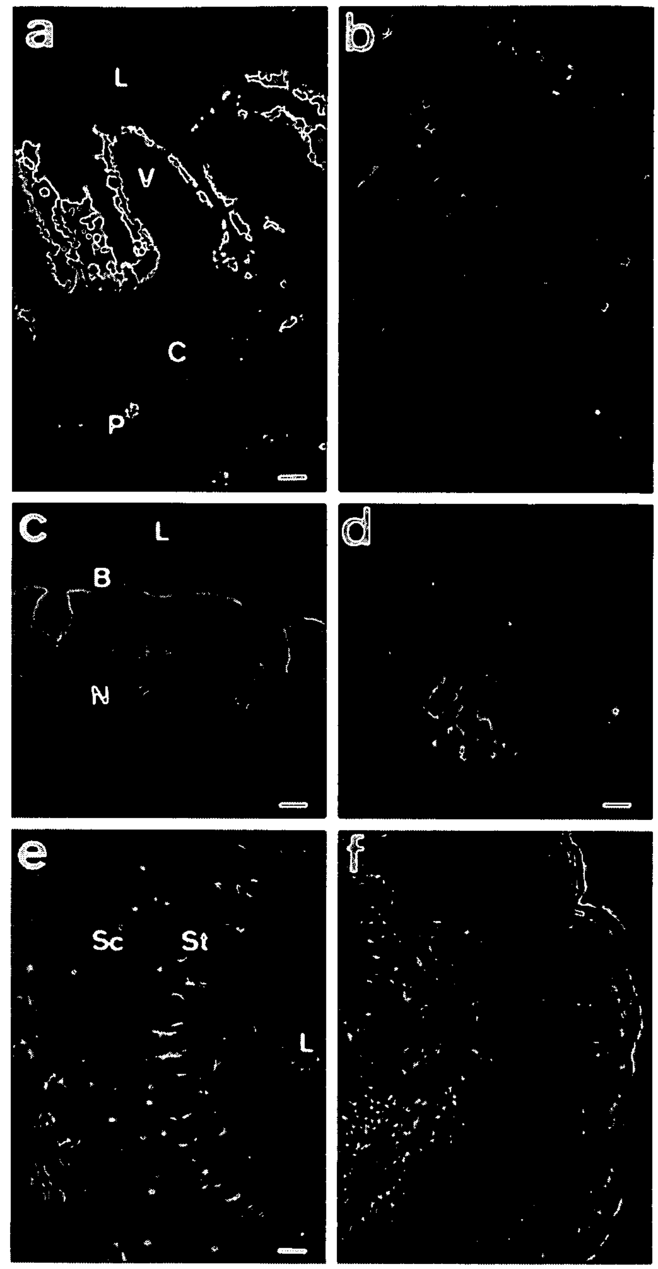


FIG. 9. **Immunofluorescence localization of PLB/LIP in rat ileum (*a*–*d*) and testis (*e* and *f*).** Thin cryosections of the ileum (*a*, *c*, and *d*) or testis (*e*) were reacted with anti-PLB/LIP antibody, while the control sections were reacted with the antibody preabsorbed with the corresponding antigen expressed in *E. coli* (*b*), and with preimmune serum (*f*) (see "Experimental Procedures"). *a*, the PLB/LIP protein located at the apical portion of the villi (*V*) and in Paneth cells (*P*). *c* and *d*, pictures of higher magnification showing brush border membranes (*B*) and Paneth cells, respectively. *c*, the high level of accumulation of the PLB/LIP protein was observed on the brush border membranes, but not in the nucleus (*N*) and on the basement membrane side. *d*, there were also immunopositive signals for PLB/LIP in granules of Paneth cells. *e*, the heads of spermatocytes (*Sc*) and spermatid (*St*) were immunopositive. *L*, lumen; *C*, crypt. *White bars* in *a*, *c*, *d*, and *e* are 60, 10, 10, and 60 μm in length, respectively.

tion of detergent solubilized PLA_2/lysophospholipase from rat intestinal brush border membranes. The use of SDS-PAGE at the final purification step gave an inactive enzyme, which renatured under appropriate conditions. That enzyme may be identical to PLB/LIP, because the amino acid composition reported was similar to that calculated from the amino acid sequence deduced in this study.

The putative signal sequences of rat PLB/LIP and AdRab-B were similar to each other. The method of von Heijne predicted cleavage between Gly^{19}-Ala^{20} for AdRabB (21); in rat PLB/LIP, the corresponding cleavage at Gly^{21}-Pro^{22} generates a new NH_2-terminal Pro, which may make this cleavage unlikely, because the occurrence of Pro at the NH_2 terminus is extremely rare. Other candidates for a signal sequence cleavage site are the peptide linkage between residues 28 and 29 or that between residues 29 and 30. It, however, remains to be clarified which

bonds are processed *in vivo*.

The results presented in this and the preceding paper (9) demonstrated that the repeat 2 is the catalytic domain that catalyzes PLA_2, lysophospholipase, and lipase activities. Comparable inhibition of these activities of purified (9), expressed full-length and repeat 2 enzymes by DFP suggested the involvement of a single active site with broad substrate specificity. This raises further questions about the functional roles of the other repeats. The primary structural similarity between repeat 2 and the other repeats, especially repeats 3 and 4 (Fig. 2*c*), implies the presence of another active sites with different specificity in these repeats, like lactase-phlorizin hydrase, which has the same structural arrangement as PLB/LIP and two distinct active sites in homologous repeat 3 and 4 (25). However, we have not yet obtained direct evidence for this. Alternatively, repeats other than repeat 2 may play roles in regulating the enzyme action of repeat 2. To address these problems, we will need the structural and functional identification of active site catalytic residues.

In rabbit lactase-phlorizin hydrolase, repeats 1 and 2 serve as parts of the propeptide that is intracellularly removed to give the mature brush border membrane form. Its precursor protein is not a zymogen but by itself is as active as the mature enzyme. In rat PLB/LIP, the intestinal enzyme was partially degraded into proteins in size ranging from 90 to 200 kDa, but the testis enzyme has the same size as the full-length PLB/LIP expressed in COS-7 cells, suggesting proteolytic cleavage did not occur in that tissue. The similar cleavage has been reported in rabbit intestinal AdRab-B (21). It is unknown at present whether this proteolysis merely reflects greater abundance of proteases in the intestinal mucosa than in the testis or whether the difference in the degree of degradation is relevant to tissue-specific roles of this enzyme. The full-length PLB/LIP is again not a zymogen because it exhibited as high a lipolytic activity as the enzyme purified from rat intestine when enzyme concentrations were estimated by immunoblot analysis (Fig. 4*a*).

We purified the 35-kDa catalytic domain of PLB/LIP with a nick at the peptide linkage between Arg^{528} and Phe^{529} that was released from brush border membrane by the action of endogenous protease(s). Although the NH_2-terminal amino acid sequences of its small and large fragments were included in the second repeat, its exact COOH-terminal processing site remained to be clarified. PLB/LIP has 14 potential *N*-glycosylation sites; repeat 2 has a single site at Asn^{696} near its COOH-terminal end. The results of concanavalin A-peroxidase stain on PVDF membrane consistently indicated that the COOH-terminal large peptide of the purified PLB/LIP was indeed glycosylated (9). The apparent molecular mass of the large peptide was about 21 kDa, which agreed with that calculated from amino acid sequence data (20.7 kDa), assuming that the processing occurs at the peptide bond just after the Lys^{710} that is present in the COOH-terminal K(R)NS sequence conserved among all repeats (Fig. 2*a*). These considerations suggested that the processing site of the purified enzyme is present at the COOH-terminal side of Asn^{696}.

In a previous study on rabbit AdRab-B, it was inferred by analogy with lactose-phlorizin hydrolase that the COOH-terminal hydrophobic domain is responsible for its membrane anchoring (21). To address the problem experimentally, we created a PLB/LIP mutant that lacks the COOH-terminal 42 amino acids including the hydrophobic stretch. The wild-type PLB/LIP was mainly found in the membrane fractions of COS-7 cells, whereas the truncated mutant was released into the culture media. Triton X-100 treatment, but not phosphatidylinositol-specific phospholipase C treatment, efficiently solubilized PLB/LIP from COS-7 cell membrane and brush border membrane fractions. These results provide evidence that the COOH-terminal hydrophobic domain does not serve as a signal to direct glycophospholipid attachment, but directly as a membrane anchor. Since there exist phospholipase C-resistant GPI-anchored proteins (26), conclusive proof for the enzyme's anchoring mechanism must, however, await structural determination of detergent-solubilized enzyme.

Surprisingly, virtually no PLA_2 and lysophospholipase activities were detectable in the culture medium of the cells expressing the deleted mutant-enzyme, despite the fact that both lipase activity and immunoreactivity were appreciably detectable. DTT treatment of the mutant, however, restored the lost activities. This can be explained as follows. The interaction between repeat 2 and another repeat(s), presumably via disulfide bridge formation, might cause the truncated mutant to undergo a conformational change, leading to selectively decreasing the binding affinity for and/or catalytic efficiency to phospholipids, of which the binding mode to the enzyme (9) and the surface quality (1) appear to differ from those of triacylglycerol. It is clear that further detailed kinetic and structural studies are required to solve this problem.

A recent study indicated that the COOH-terminal tail of lactase-phlorizin hydrolase contains the consensus sequence for the phosphorylation site by protein kinase A, and this site was indeed phosphorylated by protein kinase A *in vitro* and in tissue culture (27). This confirmed the intracellular disposition of this tail and suggested its possible physiological significance. In rat PLB/LIP, the corresponding tail does not contain the consensus sequence for a protein kinase A site. The intracellular regulation mechanism mediated by protein kinase A may not operate in the rat PLB/LIP.

Northern blot and RT-PCR analyses suggested that PLB/LIP mRNA was expressed not only in ileal mucosa but also in esophageal mucosa and testis (Figs. 6 and 7). However, rabbit AdRab-B mRNA was not detected in the testis. This may be due to species difference or due to the difference in sensitivity of the method used. The RT-PCR products of these tissues were found to be identical to one another by partial sequencing and restriction fragment length analysis. Immunoblot and immumohistochemical analyses provided further evidence for the localization of PLB/LIP protein (Figs. 8 and 9). In the ileum, a large amount of PLB/LIP existed in the brush border membrane on the apical side of villi; in contrast, secretory PLA_2s are synthesized in and secreted from cells in the bottom regions of gastrointestinal mucosa: gastric chief cells for group I PLA_2 (28) and Paneth cells for group II PLA_2 (29). A similar localization of rabbit AdRab-B has been reported (21). These results support the idea that PLB/LIP acts on the brush border membrane to facilitate the absorption of digested lipids.

Finally, immunohistochemistry showed that, in addition to the absorptive cells, Paneth cells and acrosomes of spermatids were immunopositive, but to a lesser extent, to anti-PLB/LIP antibody, suggesting PLB/LIP's roles other than intestinal digestion. Paneth cells contain a variety of antibacterial materials including group II PLA_2, which can kill certain strains of intestinal bacteria directly or with the aid of neutrophil bactericidal/permeability-increasing protein (29–31). PLB/LIP with broad specificity toward polar head groups of phospholipids actively hydrolyzes phosphatidylglycerol, the major component of bacterial cell membrane phospholipids, like group II PLA_2 (32, 33). It is interesting to examine whether PLB/LIP in Paneth cells participates in an antibacterial defense mechanism and where it is destined for after synthesis, since Paneth cells lack brush border membranes.

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Mol Genet Genomics. 2003 Apr;269(1):116-25. Epub 2003 Feb 27. Related Articles, Links

[SpringerLink FULL-TEXT ARTICLE]

The phospholipase B homolog Plb1 is a mediator of osmotic stress response and of nutrient-dependent repression of sexual differentiation in the fission yeast Schizosaccharomyces pombe.

Yang P, Du H, Hoffman CS, Marcus S.

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Although phospholipase B (PLB) enzymes have been described in eukaryotes from yeasts to mammals, their biological functions are poorly understood. Here we describe the characterization of plb1, one of five genes predicted to encode PLB homologs in the fission yeast, Schizosaccharomyces pombe. The plb1 gene is dispensable under normal growth conditions but required for viability in high-osmolarity media and for normal osmotic stress-induced gene expression. Unlike mutants defective in function for the stress-activated MAP kinase Spc1, plb1Delta cells are not hypersensitive to oxidative or temperature stresses, nor do they undergo a G2-specific arrest in response to osmotic stress. In addition to defects in osmotic stress response, plb1Delta cells exhibit a cold-sensitive defect in nutrient-mediated mating repression, a phenotype reminiscent of mutants in the cyclic AMP (cAMP) pathway. We show that, like plb1Delta cells, mutants in the cAMP pathway are defective for growth in high-osmolarity media, demonstrating a previously unrecognized role for the cAMP pathway in osmotic stress response. Furthermore, we show that gain-of function in the cAMP pathway can rescue the osmosensitive growth defect of plb1Delta cells, suggesting that the cAMP pathway is a potential downstream target of the actions of Plb1 in S. pombe.

PMID: 12715160 [PubMed - indexed for MEDLINE]

Biochem J. 2004 Jun 14;Pt. [Epub ahead of print] Related Articles, Links

Biochem J

Identification of phospholipase B from Dictyostelium discoideum reveals a new lipase family present In mammals, flies and nematodes, but not yeast.

Morgan CP, Insall R, Haynes L, Cockcroft S.

The social amoeba Dictyostelium discoideum exhibits high activities of phospholipase and lyso phospholipase (Ferber et al Eur.J.Biochem. 1970 14 253-257). We assayed Dictyostelium lysates to demonstrate the presence of a highly active phospholipase B enzyme that removed both fatty-acid chains from phosphatidylcholine (PC) and produced the water-soluble glycerophosphorylcholine. We purified the PLB activity from Dictyostelium cytosol using standard agarose media (size-exclusion and ion-exchange), and combined this with an affinity step using myristoylated ARF1, a protein which has a single fatty acid at its N-terminus. Two proteins co-purified (48kDa and 65kDa), and the 48kDa protein was digested with trypsin, peptide fragments separated by reverse phase chromatography, and the resultant peptides were sequenced by Edman degradation. From the peptide sequences obtained, database searches revealed a gene, which encodes a protein of 65 kDa with unknown function. The 48 kDa, therefore appears to be a fragment of the full length 65 kDa product. Expression of the gene in E.coli confirmed that it encodes a PLB. Characterisation of its substrate specificity indicated that in addition to PC deacylation, the enzyme also hydrolysed phosphatidylinositol and phosphatidylethanolamine. The PLB identified here is not related to existing PLBs, identified in bacteria, fungi or mammals. There are however, genes homologous to Dictyostelium PLB in mammals, flies, worms and Giardia but not in yeast. We therefore have identified a novel family of intracellular PLBs.

PMID: 15193148 [PubMed - as supplied by publisher]

THE HUMAN GENOME

Humanity has been given a great gift. With the completion of the human genome sequence, we have received a powerful tool for unlocking the secrets of our genetic heritage and for finding our place among the other participants in the adventure of life.

This week's issue of *Science* contains the report of the sequencing of the human genome from a group of authors led by Craig Venter of Celera Genomics. The report of the sequencing of the human genome from the publicly funded consortium of laboratories led by Francis Collins appears in this week's *Nature*. This stunning achievement has been portrayed—often unfairly—as a competition between two ventures, one public and one private. That characterization detracts from the awesome accomplishment jointly unveiled this week. In truth, each project contributed to the other. The inspired vision that launched the publicly funded project roughly 10 years ago reflected, and now rewards, the confidence of those who believe that the pursuit of large-scale fundamental problems in the life sciences is in the national interest. The technical innovation and drive of Craig Venter and his colleagues made it possible to celebrate this accomplishment far sooner than was believed possible. Thus, we can salute what has become, in the end, not a contest but a marriage (perhaps encouraged by shotgun) between public funding and private entrepreneurship.

A historic moment for the scientific endeavor.

There are excellent scientific reasons for applauding an outcome that has given us two winners. Two sequences are better than one; the opportunity for comparison and convergence is invaluable. Indeed, a real-world proof of the importance of access to both sets of data can be found in the pages of this issue of *Science*, in the comparative analysis by Olivier *et al.* (p. 1298).

Although we have made the point before, it is worth repeating that the sequencing of the human genome represents, not an ending, but the beginning of a new approach to biology. As Galas says in his Viewpoint (p. 1257), the knowledge that all of the genetic components of any process can be identified will give extraordinary new power to scientists. Because of this breakthrough, research can evolve from analyzing the effects of individual genes to a more integrated view that examines whole ensembles of genes as they interact to form a living human being. Several articles in this issue highlight how this approach is already beginning to revolutionize the way we look at human disease.

This has been a massive project, on a scale unparalleled in the history of biology, but of course it has built on the scientific insights of centuries of investigators. By coincidence, this landmark announcement falls during the week of the anniversary of the birth of Charles Darwin. Darwin's message that the survival of a species can depend on its ability to evolve in the face of change is peculiarly pertinent to discussions that have gone on in the past year over access to the Celera data. (Full information regarding the agreements that were reached to make the data available can be found at www.sciencemag.org/feature/data/announcement/gsp.shl.) We are willing to be flexible in allowing data repositories other than the traditional GenBank, while insisting on access to all the data needed to verify conclusions. In this domain, change is everywhere: Commercial researchers are producing more and more potentially valuable sequences, yet (at least in the United States) laws governing databases provide scant protection against piracy. Had the Celera data been kept secret, it would have been a serious loss to the scientific community. We hope that our adaptability in the face of change will enable other proprietary data to be published after peer review, in a way that satisfies our continuing commitment to full access.

It should be no surprise that an achievement so stunning, and so carefully watched, has created new challenges for the scientific venture. *Science* is proud to have played a role in bringing this discovery onto the public stage. It is literally true that this is a historic moment for the scientific endeavor. The human genome has been called the Book of Life. Rather, it is a library, in which, with rules that encourage exploration and reward creativity, we can find many of the books that will help define us and our place in the great tapestry of life.

Barbara R. Jasny and Donald Kennedy

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MEGABLAST 1.2.3-Paracel [2001-11-20]

Reference:
Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb Miller (2000),
"A greedy algorithm for aligning DNA sequences",
J Comput Biol 2000; 7(1-2):203-14.
Database: Homo_sapiens.latestgp.fa
           26,614 sequences; 200,770,258,131 total letters
Query= 1
         (4377 letters)
                                                                 Score     E
Sequences producing significant alignments:                      (bits)  Value

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AC093164.6.1.103926                                                216   9e-53
AC125617.1.1.134246                                                 90   1e-14

>AC074011.5.1.180465
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 Score =  404 bits (204), Expect = e-109
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 Strand = Plus / Plus


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 Score =  218 bits (110), Expect = 2e-53
 Identities = 110/110 (100%)
 Strand = Plus / Plus


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Query: 2426  atgacacgaatgcattcctcaatcaagctgttcccggagcaaaggctgag 2475
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 Identities = 110/110 (100%)
 Strand = Plus / Plus


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Query: 1385  ccagtcctaatgccttcttaaaccaggctgtggcaggaggccgagctgag 1434
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Sbjct: 32700 ccagtcctaatgccttcttaaaccaggctgtggcaggaggccgagctgag 32749


 Score =  216 bits (109), Expect = 9e-53
 Identities = 109/109 (100%)
 Strand = Plus / Plus


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Query: 1873  gtggttgtgcagccgttctttgaaaacgtggacatgccaaagacctcgg 1921
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 Score =  216 bits (109), Expect = 9e-53
 Identities = 109/109 (100%)
 Strand = Plus / Plus


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Sbjct: 1147  caggaagcctggaacagcctcctggcctccagcaggtacagtgagcaggagtccttcacc 1206


Query: 832   gtggttttccagcctttcttctatgagaccaccccatctctacactcgg 880
              |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1207  gtggttttccagcctttcttctatgagaccaccccatctctacactcgg 1255


 Score =  216 bits (109), Expect = 9e-53
 Identities = 109/109 (100%)
 Strand = Plus / Plus
```

```
Query: 3894  ccagcatggcatctccagtttctcctactggcaccaatacacacagcgtgaggactttgc 3953
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 91475 ccagcatggcatctccagtttctcctactggcaccaatacacacagcgtgaggactttgc 91534


Query: 3954  ggttgtggtgcagcctttcttccaaaacacactcaccccactgaacgag 4002
             |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 91535 ggttgtggtgcagcctttcttccaaaacacactcaccccactgaacgag 91583
```

```
 Score =  212 bits (107), Expect = 1e-51
 Identities = 107/107 (100%)
 Strand = Plus / Plus


Query: 2856  gagcagcatgcgcgagctggtggggtcaggccgctatgacacgcaggaggacttctctgt 2915
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 64097 gagcagcatgcgcgagctggtggggtcaggccgctatgacacgcaggaggacttctctgt 64156


Query: 2916  ggtgctgcagcccttcttccagaacatccagctccctgtcctggcgg 2962
             |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 64157 ggtgctgcagcccttcttccagaacatccagctccctgtcctggcgg 64203
```

```
 Score =  210 bits (106), Expect = 6e-51
 Identities = 106/106 (100%)
 Strand = Plus / Plus


Query: 1631  aggttcctcgggcatttgtgaacctggtgacggtgcttgagatcgtcaacctgagggagc 1690
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 41846 aggttcctcgggcatttgtgaacctggtgacggtgcttgagatcgtcaacctgagggagc 41905


Query: 1691  tgtaccaggagaaaaaagtctactgcccaaggatgatcctcaggtc 1736
             ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 41906 tgtaccaggagaaaaaagtctactgcccaaggatgatcctcaggtc 41951
```

```
 Score =  204 bits (103), Expect = 3e-49
 Identities = 103/103 (100%)
 Strand = Plus / Plus


Query: 2672  aggtgcccagagtcctggtcaacctcgtggacttcctgaaccccactatcatgcggcagg 2731
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 62262 aggtgcccagagtcctggtcaacctcgtggacttcctgaaccccactatcatgcggcagg 62321


Query: 2732  tgttcctgggaaacccagacaagtgcccagtgcagcaggccag 2774
             |||||||||||||||||||||||||||||||||||||||||||
Sbjct: 62322 tgttcctgggaaacccagacaagtgcccagtgcagcaggccag 62364
```

```
 Score =  200 bits (101), Expect = 5e-48
 Identities = 101/101 (100%)
 Strand = Plus / Plus


Query: 3428  acattctgaagaagttcaacccttacctccttggcttctctaccagcacctgggaggggga 3487
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 80322 acattctgaagaagttcaacccttacctccttggcttctctaccagcacctgggaggggga 80381


Query: 3488  cagcaggactaaatgtggcagcggaaggggccagagctagg 3528
              |||||||||||||||||||||||||||||||||||||||||
Sbjct: 80382 cagcaggactaaatgtggcagcggaaggggccagagctagg 80422



 Score =  194 bits (98), Expect = 3e-46
 Identities = 98/98 (100%)
 Strand = Plus / Plus


Query: 4001  agagaggggacactgacctcaccttcttctccgaggactgttttcacttctcagaccgcg 4060
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 92385 agagaggggacactgacctcaccttcttctccgaggactgttttcacttctcagaccgcg 92444


Query: 4061  ggcatgccgagatggccatcgcactctggaacaacatg 4098
              ||||||||||||||||||||||||||||||||||||||
Sbjct: 92445 ggcatgccgagatggccatcgcactctggaacaacatg 92482



 Score =  192 bits (97), Expect = 1e-45
 Identities = 97/97 (100%)
 Strand = Plus / Plus


Query: 2961  ggatgggctcccagatacgtccttctttgccccagactgcatccacccaaatcagaaatt 3020
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 65295 ggatgggctcccagatacgtccttctttgccccagactgcatccacccaaatcagaaatt 65354


Query: 3021  ccactcccagctggccagagccctttggaccaatatg 3057
              |||||||||||||||||||||||||||||||||||||
Sbjct: 65355 ccactcccagctggccagagccctttggaccaatatg 65391



 Score =  192 bits (97), Expect = 1e-45
 Identities = 97/97 (100%)
 Strand = Plus / Plus


Query: 1920  ggaaggattgcctgacaactctttcttcgctcctgactgtttccacttcagcagcaagtc 1979
```

```
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 49117 ggaaggattgcctgacaactctttcttcgctcctgactgtttccacttcagcagcaagtc 49176


Query: 1980  tcactcccgagcagccagtgctctctggaacaatatg 2016
             |||||||||||||||||||||||||||||||||||||
Sbjct: 49177 tcactcccgagcagccagtgctctctggaacaatatg 49213



 Score =  188 bits (95), Expect = 2e-44
 Identities = 95/95 (100%)
 Strand = Plus / Plus


Query: 3725  agctcccaagggctttcgtcaacgtggtggaggtcatggagctggctagcctgtaccagg 3784
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 88535 agctcccaagggctttcgtcaacgtggtggaggtcatggagctggctagcctgtaccagg 88594


Query: 3785  gccaaggcgggaaatgtgccatgctggcagctcag 3819
             |||||||||||||||||||||||||||||||||||
Sbjct: 88595 gccaaggcgggaaatgtgccatgctggcagctcag 88629



 Score =  170 bits (86), Expect = 5e-39
 Identities = 86/86 (100%)
 Strand = Plus / Plus


Query: 1731  caggtctctgtgtccctgtgtcctgaagtttgatgataactcaacagaacttgctaccct 1790
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 45201 caggtctctgtgtccctgtgtcctgaagtttgatgataactcaacagaacttgctaccct 45260


Query: 1791  catcgaattcaacaagaagtttcagg 1816
             ||||||||||||||||||||||||||
Sbjct: 45261 catcgaattcaacaagaagtttcagg 45286



 Score =  168 bits (85), Expect = 2e-38
 Identities = 85/85 (100%)
 Strand = Plus / Plus


Query: 2772  cagcgttttgtgtaactgcgttctgaccctgcgggagaactcccaagagctagccaggct 2831
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 63406 cagcgttttgtgtaactgcgttctgaccctgcgggagaactcccaagagctagccaggct 63465


Query: 2832  ggaggccttcagccgagcctaccgg 2856
             |||||||||||||||||||||||||
Sbjct: 63466 ggaggccttcagccgagcctaccgg 63490
```

```
Score =  167 bits (84), Expect = 7e-38
Identities = 84/84 (100%)
Strand = Plus / Plus


Query: 1485  gaggatacactttcaggaagactggaagataataaccctgtttataggcggcaatgacct 1544
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 39069 gaggatacactttcaggaagactggaagataataaccctgtttataggcggcaatgacct 39128


Query: 1545  ctgtgatttctgcaatgatctggt 1568
              ||||||||||||||||||||||||
Sbjct: 39129 ctgtgatttctgcaatgatctggt 39152



Score =  167 bits (84), Expect = 7e-38
Identities = 88/89 (98%), Gaps = 1/89 (1%)
Strand = Plus / Plus


Query: 3580  gacatcaacctggagaaagactggaagctggtcacactcttcattggggtcaacgacttg 3639
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 85871 gacatcaacctggagaaagactggaagctggtcacactcttcattggggtcaacgacttg 85930


Query: 3640  tgtcattactgtgagaatccgg-aggccc 3667
              |||||||||||||||||||||| ||||||
Sbjct: 85931 tgtcattactgtgagaatccggtaggccc 85959



Score =  165 bits (83), Expect = 3e-37
Identities = 83/83 (100%)
Strand = Plus / Plus


Query: 1006 caggagagcccctatctgttcagctacagaaacagcaactacctgaccagactgcagaaa 1065
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 9450 caggagagcccctatctgttcagctacagaaacagcaactacctgaccagactgcagaaa 9509


Query: 1066 ccccaagacaagcttgaggtaag 1088
             |||||||||||||||||||||||
Sbjct: 9510 ccccaagacaagcttgaggtaag 9532



Score =  161 bits (81), Expect = 5e-36
Identities = 81/81 (100%)
Strand = Plus / Plus


Query: 2527  agagtaaatttccatgaagactggaaggtcatcacagtgctgatcggaggcagcgattta 2586
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 60695 agagtaaatttccatgaagactggaaggtcatcacagtgctgatcggaggcagcgattta 60754
```

```
Query: 2587  tgtgactactgcacagattcg 2607
             |||||||||||||||||||||
Sbjct: 60755 tgtgactactgcacagattcg 60775


 Score =  157 bits (79), Expect = 7e-35
 Identities = 79/79 (100%)
 Strand = Plus / Plus


Query: 3819  gaacaactgcacttgcctcagacactcgcaaagctccctggagaagcaagaactgaagaa 3878
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 90029 gaacaactgcacttgcctcagacactcgcaaagctccctggagaagcaagaactgaagaa 90088


Query: 3879  agtgaactggaacctccag 3897
             |||||||||||||||||||
Sbjct: 90089 agtgaactggaacctccag 90107


 Score =  153 bits (77), Expect = 1e-33
 Identities = 81/82 (98%), Gaps = 1/82 (1%)
 Strand = Plus / Plus


Query: 1199  ctctcacggcaggcaatggggccgggtccacacctgggaacgtcttggacgtcttgactc 1258
             |||||| |||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 24716 ctctca-ggcaggcaatggggccgggtccacacctgggaacgtcttggacgtcttgactc 24774


Query: 1259  agtaccgaggcctgtcctggag 1280
             ||||||||||||||||||||||
Sbjct: 24775 agtaccgaggcctgtcctggag 24796


 Score =  153 bits (77), Expect = 1e-33
 Identities = 77/77 (100%)
 Strand = Plus / Plus


Query: 4098   gctggaaccagtgggccgcaagactacctccaacaacttcacccacagccgagccaaact 4157
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 100368 gctggaaccagtgggccgcaagactacctccaacaacttcacccacagccgagccaaact 100427


Query: 4158   caagtgcccctctcctg 4174
              |||||||||||||||||
Sbjct: 100428 caagtgcccctctcctg 100444


 Score =  149 bits (75), Expect = 2e-32
```

```
 Identities = 75/75 (100%)
 Strand = Plus / Plus


Query: 2016  gctggagcctgttggccagaagacgactcgtcataagtttgaaaacaagatcaatatcac 2075
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 49447 gctggagcctgttggccagaagacgactcgtcataagtttgaaaacaagatcaatatcac 49506


Query: 2076  atgtccgaaccaggt 2090
             |||||||||||||||
Sbjct: 49507 atgtccgaaccaggt 49521



 Score =  147 bits (74), Expect = 7e-32
 Identities = 74/74 (100%)
 Strand = Plus /. Plus


Query: 936   gatggagccagcaggagagaaagatgagccattgagtgtaaaacacgggaggccaatgaa 995
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 8302  gatggagccagcaggagagaaagatgagccattgagtgtaaaacacgggaggccaatgaa 8361


Query: 996   gtgtccctctcagg 1009
             ||||||||||||||
Sbjct: 8362  gtgtccctctcagg 8375



 Score =  147 bits (74), Expect = 7e-32
 Identities = 74/74 (100%)
 Strand = Plus / Plus


Query: 2248  gctggcaatggaattggctccaaaccagacgacctccccgatgtcaccacacagtatcgg 2307
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 53124 gctggcaatggaattggctccaaaccagacgacctccccgatgtcaccacacagtatcgg 53183


Query: 2308  ggactgtcatacag 2321
             ||||||||||||||
Sbjct: 53184 ggactgtcatacag 53197



 Score =  145 bits (73), Expect = 3e-31
 Identities = 73/73 (100%)
 Strand = Plus / Plus


Query: 3057  gcttgaaccacttggaagcaaaacagagaccctggacctgagagcagagatgcccatcac 3116
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 65938 gcttgaaccacttggaagcaaaacagagaccctggacctgagagcagagatgcccatcac 65997
```

```
Query: 3117  ctgtcccactcag 3129
             |||||||||||||
Sbjct: 65998 ctgtcccactcag 66010
```

Score = 137 bits (69), Expect = 7e-29
Identities = 69/69 (100%)
Strand = Plus / Plus

```
Query: 1566  ggtccactattctccccagaacttcacagacaacattggaaaggccctggacatcctcca 1625
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 41510 ggtccactattctccccagaacttcacagacaacattggaaaggccctggacatcctcca 41569

Query: 1626  tgctgaggt 1634
             |||||||||
Sbjct: 41570 tgctgaggt 41578
```

Score = 135 bits (68), Expect = 3e-28
Identities = 68/68 (100%)
Strand = Plus / Plus

```
Query: 3316  acagcagtgggagctcgaccaaacaactccagtgacctacccacatcttggaggggactc 3375
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 79708 acagcagtgggagctcgaccaaacaactccagtgacctacccacatcttggaggggactc 79767

Query: 3376  tcttggag 3383
             ||||||||
Sbjct: 79768 tcttggag 79775
```

Score = 135 bits (68), Expect = 3e-28
Identities = 68/68 (100%)
Strand = Plus / Plus

```
Query: 3128  agaatgagcccttcctgagaacccctcggaatagtaactacacgtaccccatcaagccag 3187
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 73472 agaatgagcccttcctgagaacccctcggaatagtaactacacgtaccccatcaagccag 73531

Query: 3188  ccattgag 3195
             ||||||||
Sbjct: 73532 ccattgag 73539
```

Score = 133 bits (67), Expect = 1e-27
Identities = 67/67 (100%)
Strand = Plus / Plus

```
Query: 3660  ggaggcccacttggccacggaatatgttcagcacatccaacaggccctggacatcctctc 3719
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 88005 ggaggcccacttggccacggaatatgttcagcacatccaacaggccctggacatcctctc 88064


Query: 3720  tgaggag 3726
             |||||||
Sbjct: 88065 tgaggag 88071
```

```
 Score =  131 bits (66), Expect = 4e-27
 Identities = 69/70 (98%)
 Strand = Plus / Plus


Query: 2607  gaatctgtattctgcagccaactttgttgaccatctccgcaatgccttggacgtcctgca 2666
             ||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct: 61341 gaatctgtattctgcagccaactttgttcaccatctccgcaatgccttggacgtcctgca 61400


Query: 2667  tagagaggtg 2676
             ||||||||||
Sbjct: 61401 tagagaggtg 61410
```

```
 Score =  131 bits (66), Expect = 4e-27
 Identities = 66/66 (100%)
 Strand = Plus / Plus


Query: 1082  aggtaagagaaggagcggaaatcagatgtcctgacaaagacccctccgatacggttccca 1141
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 12369 aggtaagagaaggagcggaaatcagatgtcctgacaaagacccctccgatacggttccca 12428


Query: 1142  cctcag 1147
             ||||||
Sbjct: 12429 cctcag 12434
```

```
 Score =  125 bits (63), Expect = 3e-25
 Identities = 63/63 (100%)
 Strand = Plus / Plus


Query: 2186  cagtgcatgccctgagacctgcagacatccaagttgtggctgctctgggggattctctga 2245
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 52101 cagtgcatgccctgagacctgcagacatccaagttgtggctgctctgggggattctctga 52160


Query: 2246  ccg 2248
             |||
```

```
Sbjct: 52161 ccg 52163


 Score =  125 bits (63), Expect = 3e-25
 Identities = 63/63 (100%)
 Strand = Plus / Plus


Query: 2126   agggtcatgggacctggctgccatgcagggacagagccccttctgccttgcaccctacct 2185
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 51141 agggtcatgggacctggctgccatgcagggacagagccccttctgccttgcaccctacct 51200


Query: 2186   cag 2188
              |||
Sbjct: 51201 cag 51203


 Score =  125 bits (63), Expect = 3e-25
 Identities = 63/63 (100%)
 Strand = Plus / Plus


Query: 3194   agaactggggcagtgacttcctgtgtacagagtggaaggcttccaatagtgttccaacct 3253
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 77168 agaactggggcagtgacttcctgtgtacagagtggaaggcttccaatagtgttccaacct 77227


Query: 3254   ctg 3256
              |||
Sbjct: 77228 ctg 77230


 Score =  123 bits (62), Expect = 1e-24
 Identities = 62/62 (100%)
 Strand = Plus / Plus


Query: 1146   agttcataggctgaagccggctgacatcaacgtaattggagccctgggtgactctctcac 1205
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 22482 agttcataggctgaagccggctgacatcaacgtaattggagccctgggtgactctctcac 22541


Query: 1206   gg 1207
              ||
Sbjct: 22542 gg 22543


 Score =  119 bits (60), Expect = 2e-23
 Identities = 60/60 (100%)
 Strand = Plus / Plus
```

```
Query: 3256  gtccaccagctccgaccagcagacatcaaagtggtggccgccctgggtgactctctgact 3315
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 77783 gtccaccagctccgaccagcagacatcaaagtggtggccgccctgggtgactctctgact 77842
```

Score = 115 bits (58), Expect = 2e-22
Identities = 58/58 (100%)
Strand = Plus / Plus

```
Query: 879   ggaggacccccgactccaggattctaccacgctggcctggcatctctggaataggatg 936
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2654  ggaggacccccgactccaggattctaccacgctggcctggcatctctggaataggatg 2711
```

Score = 113 bits (57), Expect = 1e-21
Identities = 57/57 (100%)
Strand = Plus / Plus

```
Query: 3524  ctagggacatgccagcccaggcctgggacctggtagagcgaatgaaaaacagccccg 3580
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 84496 ctagggacatgccagcccaggcctgggacctggtagagcgaatgaaaaacagccccg 84552
```

Score = 103 bits (52), Expect = 9e-19
Identities = 52/52 (100%)
Strand = Plus / Plus

```
Query: 2475  ggatcttatgagccaagtccaaactctgatgcagaagatgaaagatgatcat 2526
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 60252 ggatcttatgagccaagtccaaactctgatgcagaagatgaaagatgatcat 60303
```

Score = 103 bits (52), Expect = 9e-19
Identities = 52/52 (100%)
Strand = Plus / Plus

```
Query: 1434  ggatctacctgtccaggccaggaggctggtggacctgatgaagaatgacacg 1485
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 37550 ggatctacctgtccaggccaggaggctggtggacctgatgaagaatgacacg 37601
```

Score = 97.6 bits (49), Expect = 6e-17
Identities = 49/49 (100%)
Strand = Plus / Plus

```
Query: 2317  tacagtgcaggaggggacggctccctggagaatgtgaccaccttaccta 2365
             |||||||||||||||||||||||||||||||||||||||||||||||||
```

```
Sbjct: 57443 tacagtgcaggaggggacggctccctggagaatgtgaccaccttaccta 57491


 Score = 91.7 bits (46), Expect = 4e-15
 Identities = 46/46 (100%)
 Strand = Plus / Plus


Query: 3382  agcattggaggggatgggaacttggagactcacaccacactgccca 3427
              ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 80041 agcattggaggggatgggaacttggagactcacaccacactgccca 80086


 Score = 91.7 bits (46), Expect = 4e-15
 Identities = 46/46 (100%)
 Strand = Plus / Plus


Query: 1279  agcgtcggcggagatgagaacatcggcaccgttaccaccctggcga 1324
              ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 26237 agcgtcggcggagatgagaacatcggcaccgttaccaccctggcga 26282


 Score = 85.7 bits (43), Expect = 2e-13
 Identities = 43/43 (100%)
 Strand = Plus / Plus


Query: 2086  caggtccagccgtttctgaggacctacaagaacagcatgcagg 2128
              |||||||||||||||||||||||||||||||||||||||||||
Sbjct: 50573 caggtccagccgtttctgaggacctacaagaacagcatgcagg 50615


>AC093164.6.1.103926
          Length = 103926

 Score =  216 bits (109), Expect = 9e-53
 Identities = 109/109 (100%)
 Strand = Plus / Plus


Query: 772    caggaagcctggaacagcctcctggcctccagcaggtacagtgagcaggagtccttcacc 831
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 103073 caggaagcctggaacagcctcctggcctccagcaggtacagtgagcaggagtccttcacc 103132


Query: 832    gtggttttccagcctttcttctatgagaccaccccatctctacactcgg 880
               |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 103133 gtggttttccagcctttcttctatgagaccaccccatctctacactcgg 103181


 Score =  184 bits (93), Expect = 3e-43
```

```
 Identities = 93/93 (100%)
 Strand = Plus / Plus


Query: 324   agtcctttcagacatcatcagatatttcagtccttctgttccaatgcctgtgtgccacac 383
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 90684 agtcctttcagacatcatcagatatttcagtccttctgttccaatgcctgtgtgccacac 90743


Query: 384   tggaaagagagtcataccccacgatggtgctga 416
             |||||||||||||||||||||||||||||||||
Sbjct: 90744 tggaaagagagtcataccccacgatggtgctga 90776


 Score =  174 bits (88), Expect = 3e-40
 Identities = 88/88 (100%)
 Strand = Plus / Plus


Query: 468   gcaacttgactttcaatttgactggaagctcatcaatgtgttcttcagtaatgcaagcca 527
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 93476 gcaacttgactttcaatttgactggaagctcatcaatgtgttcttcagtaatgcaagcca 93535


Query: 528   gtgttacctgtgcccctctgctcaacag 555
             ||||||||||||||||||||||||||||
Sbjct: 93536 gtgttacctgtgcccctctgctcaacag 93563


 Score =  163 bits (82), Expect = 1e-36
 Identities = 82/82 (100%)
 Strand = Plus / Plus


Query: 617    aggtccccagagcatttgtaaacctggtggacctctctgaggttgcagaggtctctcgtc 676
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 100466 aggtccccagagcatttgtaaacctggtggacctctctgaggttgcagaggtctctcgtc 100525


Query: 677    agtatcacggcacttggctcag 698
              ||||||||||||||||||||||
Sbjct: 100526 agtatcacggcacttggctcag 100547


 Score =  159 bits (80), Expect = 2e-35
 Identities = 80/80 (100%)
 Strand = Plus / Plus


Query: 696    cagccctgcaccagagccctgtaattgctcagaggagaccacccggctggccaaggtggt 755
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 101732 cagccctgcaccagagccctgtaattgctcagaggagaccacccggctggccaaggtggt 101791
```

```
Query: 756    gatgcagtggtcttatcagg 775
              ||||||||||||||||||||
Sbjct: 101792 gatgcagtggtcttatcagg 101811
```

```
 Score =  137 bits (69), Expect = 7e-29
 Identities = 69/69 (100%)
 Strand = Plus / Plus
```

```
Query: 116   agaccctgaagaattctccattcccatgcaacccaaataaattaggagtgaatatgcctt 175
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 79833 agaccctgaagaattctccattcccatgcaacccaaataaattaggagtgaatatgcctt 79892

Query: 176   ctaaatcag 184
             |||||||||
Sbjct: 79893 ctaaatcag 79901
```

```
 Score =  137 bits (69), Expect = 7e-29
 Identities = 69/69 (100%)
 Strand = Plus / Plus
```

```
Query: 552   acagaatgggcttgcggcgggcggcgtggatgagctgatgggggtgctggactacctgca 611
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 99684 acagaatgggcttgcggcgggcggcgtggatgagctgatgggggtgctggactacctgca 99743

Query: 612   gcaggaggt 620
             |||||||||
Sbjct: 99744 gcaggaggt 99752
```

```
 Score =  127 bits (64), Expect = 6e-26
 Identities = 64/64 (100%)
 Strand = Plus / Plus
```

```
Query: 54    agggacccctcagatccatacctctcctagaaagagtacattggaagggcagctatggcc 113
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 78175 agggacccctcagatccatacctctcctagaaagagtacattggaagggcagctatggcc 78234

Query: 114   agag 117
             ||||
Sbjct: 78235 agag 78238
```

```
 Score =  123 bits (62), Expect = 1e-24
 Identities = 62/62 (100%)
 Strand = Plus / Plus
```

```
Query: 182   cagttcactctctgaagccttctgatattaaatttgtggcagccattggcaatctggaaa 241
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 81071 cagttcactctctgaagccttctgatattaaatttgtggcagccattggcaatctggaaa 81130

Query: 242   tt 243
             ||
Sbjct: 81131 tt 81132


 Score =  111 bits (56), Expect = 4e-21
 Identities = 56/56 (100%)
 Strand = Plus / Plus

Query: 1     atggggctgcggccaggcattttcctcctggagctgctgctgcttctggggcaagg 56
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 57484 atggggctgcggccaggcattttcctcctggagctgctgctgcttctggggcaagg 57539


 Score =  103 bits (52), Expect = 9e-19
 Identities = 52/52 (100%)
 Strand = Plus / Plus

Query: 417   agacttgtggattcaggctcaagaactggtgagaaacatgaaagagaacctg 468
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 91097 agacttgtggattcaggctcaagaactggtgagaaacatgaaagagaacctg 91148


 Score = 83.8 bits (42), Expect = 9e-13
 Identities = 42/42 (100%)
 Strand = Plus / Plus

Query: 284   ggactgaaaggccacagcaggtgtgcatgggagtgatgacag 325
             ||||||||||||||||||||||||||||||||||||||||||
Sbjct: 87273 ggactgaaaggccacagcaggtgtgcatgggagtgatgacag 87314


 Score = 83.8 bits (42), Expect = 9e-13
 Identities = 42/42 (100%)
 Strand = Plus / Plus

Query: 244   cctccagacccagggacgggcgatctggagaagcaagactgg 285
             ||||||||||||||||||||||||||||||||||||||||||
Sbjct: 86636 cctccagacccagggacgggcgatctggagaagcaagactgg 86677
```

```
>AC125617.1.1.134246
          Length = 134246

 Score = 89.7 bits (45), Expect = 1e-14
 Identities = 73/82 (89%), Gaps = 2/82 (2%)
 Strand = Plus / Plus


Query: 3733  agggctttcgtcaacgtggtggaggtcatggagct-ggctagcctgtaccagggccaagg 3791
              |||| ||| ||| |||||||||||||||||||| | ||| |||||| |||||||||||||
Sbjct: 68287 agggttttgtccacgtggtggaggtcatggag-tgggccagcctgcaccagggccaagg 68345


Query: 3792  cgggaaatgtgccatgctggca 3813
               ||||||||| |||||||||||
Sbjct: 68346 tgggaaatgtaccatgctggca 68367
```